ANNUAL REPORT OF THE SCIENTIFIC DIRECTOR
National Heart, Lung, and Blood Institute
October 1, 1991 to September 30, 1992

A scientific program as large and diversified as that of the NHLBI Division of Intramural Research cannot be summarized in a few pages. Perhaps what can be done by appropriately chosen examples is to capture its flavor and to highlight a few selected accomplishments from among the many that are described in the pages that follow.

For a number of years, the DIR has been of relatively constant size with about 70 tenured, doctoral-level scientists, a total staff of about 550 and about 150 doctoral-level guest scientists. The DIR occupies about 132,000 square feet of space in 6 buildings and clinical investigators have the use of about 80 beds in 4 different patient care units. The total budget is about $97,000,000 which includes about $29,000,000 for laboratory operating expenses and equipment purchases and about $29,000,000 to support clinical research.

This year a number of major renovations of laboratory space were completed or are in progress, including about 5 laboratories in Building 3 to house an expanded NMR facility for the study of the 3-dimensional structure of macromolecules, about 30 laboratories and administrative offices in Building 10, most of which had not been renovated since their initial occupancy in 1953-1954, and the conversion of a patient care unit into the first research bone marrow transplantation unit at the NIH, which should open early in 1993. In addition, construction is now underway for about 16,000 square feet of laboratory and animal holding space in a rented building off campus. This will allow significant expansion of animal research, especially with non-human primates, as part of the Institute's expanding efforts toward developing genetic therapy for a number of pulmonary, cardiovascular and blood diseases.

Although the DIR remains essentially unchanged organizationally, the orientation of the research continues to move at an ever increasing pace towards macromolecular structure, molecular biology and genetics and molecular medicine including, as mentioned, gene therapy although more traditional clinical research is still, and will continue to be, well supported. In this context, the resignation of Dr. W. French Anderson - who left the Institute to join his wife who accepted a major position in Los Angeles - should be noted. Fortunately, Dr. Anderson has left a cadre of well-trained investigators who will continue the research he initiated and other Branch Chiefs have followed his lead in establishing their own independent programs.

Research in the **PULMONARY BRANCH** centers on chronic hereditary and acquired disorders of the lung. The strong focus this year has been on cystic fibrosis (CF), an autosomal recessive disorder resulting from mutations in the cystic fibrosis transmembrane regulator (CFTR) gene. Studies include the control of expression of the CFTR gene, development of an adenovirus vector for potential gene therapy of the pulmonary manifestations of the disease and demonstration that delivery by aerosolization of human, recombinant DNase significantly reduces the viscosity of lung secretions

(by cleaving DNA that is largely responsible for the high viscosity) and improves lung function.

The **CARDIOLOGY BRANCH** has recently initiated a molecular and cell biology program to complement its long-standing strengths in physiological and clinical studies. One area in which this combined approach has been undertaken is the problem of coronary restenosis in which smooth muscle cells proliferate in response to vascular injury accompanying coronary angioplasty.

Clinical studies have identified several specific mechanisms that could be responsible for sudden death in hypertrophic cardiomyopathy (HCM) patients. Patients with obstructive HCM have been effectively treated by dual chamber pacing which has a number of advantages over the traditional surgical approaches (lower cost, lower morbidity and lower mortality). Scientists in the **CARDIOLOGY BRANCH** and **CLINICAL HEMATOLOGY BRANCH** have made major progress in understanding the genetic basis of HCM, in correlating two mutations of the myosin heavy chain with the clinical severity of the disease and in demonstrating biochemical deficiencies of the mutant myosins in *in vitro* assays.

In addition to the studies on HCM, examples of research in the **CLINICAL HEMATOLOGY BRANCH** include: developing techniques to promote division of stem cells by a combination of interleukin-3, interleukin-6 and stem cell factor; insertion of the genes for adenosine deaminase and human multi-drug resistance into repopulating primate stem cells in pre-clinical studies; description of a human cell line that supports B19 parvovirus propagation; B19 parvovirus vaccine development with a baculovirus protein expression system as source of the immunizing protein.

The **MOLECULAR DISEASE BRANCH** continues to study the roles of plasma lipoproteins in lipid transport in normal individuals and patients with elevated levels of plasma cholesterol and triglycerides. In a search for pharmacologic agents that might reduce the production of apoB (apoB-containing LDL is a major risk factor for cardiovascular disease), vanadate has been shown to cause a 80% decrease in apoB mRNA and a 90% decrease in apoB protein production in a human cell line with a 3-fold increase in the fraction of apoB-48. Insights into the hydrolysis of lipoprotein triglycerides by lipoprotein lipase have been gained by structure-function studies on enzymes produced by site-directed mutagenesis and chimeric proteins combining functional domains of lipoprotein lipase, hepatic lipase and pancreatic lipase.

This year, studies in the **PATHOLOGY BRANCH** include the amount of coronary narrowing in patients with abdominal aortic aneurysm and a comparison of the atherosclerotic plaques in men and women. The drug ICRF 187 has been shown to protect against the cardiotoxicity of doxorubicin, a highly effective antineoplastic agent and the morphology of the cardiotoxicity of amiodarone and interleukin-2 have been studied.

Recent studies in the **HYPERTENSION-ENDOCRINE BRANCH** have shown that a recently discovered natriuretic peptide, urodilatin, is more effective than atrial natriuretic factor in the treatment of cardiac edema and that therapy is improved by the addition of an inhibitor of neutral endopeptidase which degrades the active peptides.

Major accomplishments of the **MOLECULAR HEMATOLOGY BRANCH** this year include: a successful human gene therapy clinical trial for adenosine deaminase deficiency; initiation of several human gene therapy protocols for the treatment of advanced cancer; progress in the preclinical work required for gene therapy protocols for AIDS, hemophilia, and cardiovascular disease; and continued progress in understanding the regulation of gene expression through studies of the promotor elements of adenovirus 2 major late transcription unit and the eukaryotic translation factor eIF-2 alpha gene.

The **LABORATORY OF BIOCHEMICAL GENETICS** is intensely studying mouse and *Drosophila* homeobox and Pou-domain genes (the protein products of these genes bind to DNA and regulate gene expression). Several mouse homeobox and Pou-domain genes have been cloned, sequenced, mapped to their respective chromosomes and their tissue expression established. Expression of the *Drosophila* homeobox gene NK-2 commits cells to a neuroblast pathway. Three-dimensional structural studies of the NK-2 homeodomain have begun in order to determine the basis of its interaction with DNA. Others areas of activity include the regulation of a gene for a voltage-sensitive calcium subunit, regulation of the proenkaphlin and proneuropeptide Y genes and the study of the distribution of tropomodulin (a novel tropomyosin-binding protein) in myofibrils.

Highlights of this year's research by the **LABORATORY OF BIOCHEMISTRY** include studies on the oxidation of proteins by lipid oxidation products of polyunsaturated fatty acids; continued research on signal transduction pathways involving phospholipase C; metal-catalyzed oxidative modification of proteins implicated in the aging process and a variety of diseases as well as in normal processes; in metabolism of the trace metal selenium which is incorporated into proteins as selenocysteine and nucleic acids as selenouridine - importantly the highly reactive selenium donor in the biosynthesis of these compounds has been identified as selenophosphate; and many other investigations of regulatory pathways and protein structure-function relationships.

The **BIOPHYSICAL CHEMISTRY LABORATORY** has largely shifted from a study of small organic molecules of pharmacologic interest to the study of macromolecules. A newly acquired 600 Mhz NMR system is used for the study of the structure of a homeobox gene product and the gp-120 envelope glycoproteins of HIV-1. Mass spectroscopic capability is in place for the determination of molecular weights of protein, carbohydrate and lipid macromolecules as well as protein sequencing.

Research in the **CARDIAC ENERGETICS LABORATORY** is currently heavily oriented towards the use of NMR as non-invasive means for studying physiological processes at the molecular level *in vivo*. Current interests include: the regulation of coronary blood flow through the ATP-sensitive potassium channel; possible control of oxidative phosphorylation in the intact heart by the redox state of mitochondrial NADH; development of new imaging techniques based on the principle of magnetization transfer between protons on macromolecules and water protons in intact tissues; the application of one of only 3 existing 4-Tesla 1-meter bore NMR systems for human studies, including imaging and metabolic studies; the successful use of MRI to determine tissue oxygen levels, more specifically, at the present time, blood oxygenation levels.

The molecular basis of cell motility continues to be one of the major interests of the **LABORATORY OF CELL BIOLOGY**. Studies of non-muscle myosin I and myosin II have resulted in considerable insight into the biochemical mechanisms and structural basis of regulation of actomyosin ATPase activity by heavy chain phosphorylation. Genetic studies have provided new insights into the possible roles of some of the myosin I isoforms in *Dictyostelium* and immunoelectron microscopy had identified the different intracellular localizations of the myosin I isoforms in *Acanthamoeba*. New insights on the mechanism of ATP hydrolysis by 70-kDa heat shock proteins have been gained. Further advances on time-resolved fluorescence spectroscopic techniques for studying macromolecular structure have occurred. A new intratracheal pulmonary ventilation system was devised.

Much of the effort of the **LABORATORY OF CELLULAR METABOLISM** this year was focussed on the ADP-ribosylation factors (ARFs) that are involved in intracellular vesicle transport from the endoplasmic reticulum to Golgi. This work has included cloning and expression to evaluate structure-function relationships and effects of post-translational modifications (myristoylation) and the interactions of the individual ARF isoforms with Golgi *in vitro*. Other work included the cloning and sequencing of two different cyclic GMP-inhibited phosphodiesterase (enzymes that degrade CAMP and CGMP).

Scientists in the **LABORATORY OF CHEMICAL PHARMACOLOGY** are studying the mechanisms by which antigen-binding to receptor-bound IgE induces degranulation of mast cells. Current focus is on the role of protein kinase C isozymes with the finding that protein kinase C beta is the major player. In other research, all of the major neoantigens (i.e. trifluoro-acetylated proteins) produced by halothane, and which may be responsible for the hypersensitive reaction leading to liver necrosis, have been identified. Work on metabolic alterations of heme proteins, the action of nitric oxide and mechanisms of action of cytochrome P-450 enzymes continues.

The continuing goal of the **LABORATORY OF KIDNEY AND ELECTROLYTE METABOLISM** is to understand the function of the kidney. This year, among other studies, a number of hormones were screened for their ability to regulate CAMP formation in the inner medullary collecting duct in order to understand regulation of transport of water, urea and salt. Studies of cell shape change by computer-controlled, video-enhanced light microscopy as a measure of water permeability continue. The biochemistry and physiology of the organic osmolytes sorbitol, inositol, glycerol-phosphorylcholine and betaine that regulate intracellular osmolality of renal inner medullary cells exposed to a an interstitial fluid of variable hyperosmolality is a major focus of this Laboratory.

The **LABORATORY OF MOLECULAR CARDIOLOGY** studies the regulation, expression, and function of contractile proteins in muscle and non-muscle cells. This year the functional properties of a mutant myosin from muscle of hypertrophic cardiomyopathy patients were studied; a new insert between the ATP and actin binding sites in the head of non-muscle and smooth muscle myosin was discovered; the consequences of a 7 amino acid insertion in the head domain of aortic smooth muscle myosin was studied; and a new program was initiated to study the function and regulation of 3 homeobox genes that appear to have a role in muscle differentiation in developing *Drosophila*.

Research in the **SECTION ON PULMONARY AND MOLECULAR IMMUNOLOGY** centers on a few proteins critical to T-cell activation. The structure and function of the interleukin-2 (IL-2) receptor (whose expression is induced by antigens and mitogens) has been a longstanding interest of this group. Current research includes aspects of the structure, function and molecular regulation of two of the three major proteins of the IL-2 receptor, IL-2Ralpha and IL-2Rbeta. This work includes the discovery of a new enhancer in the IL-2Ralpha gene and two distinct enhancer regions for IL-2Rbeta have been mapped. Act-2, a 66-amino acid cytokine secreted by activated T-cells (and which was discovered by this group) continues to be studied including the genomic organization and chromosomal localization of the Act-2 gene.

Annual Report of the Section on Pulmonary and Molecular Immunology
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In the past year, the research of the Section on Pulmonary and Molecular Immunology has centered on the study of select proteins critical to the process of T-cell activation. T-cells play a central role in mounting an immune response and as such are intimately related to components of each of the three principal disease area focuses of NHLBI. This report summarizes studies related to (1) IL-2 receptor structure and function (2) Molecular regulation of IL-2 receptor α and β chain genes and related work on NF-κB, and (3) The Act-2 cytokine. The IL-2 receptor has been a longstanding focus of this group, members of which were the first to discover the existence of both the IL-2Rα and IL-2Rβ chains and to clone cDNAs encoding the IL-2Rα chain. Research in this area relates to components of structure, function, and molecular regulation of the IL-2Rα and IL-2Rβ chain genes. Because of the critical role of the NF-κB binding site in the IL-2Rα chain gene in mediating induction in response to HTLV-I infection, specific aspects of this transcription factor have been studied. In addition, the group has focused some effort on a new cytokine, denoted Act-2, that it was the first to discover in 1988. Each of these areas will be discussed in turn.

IL-2 receptor-- structure and function.

The human interleukin-2 receptor is being studied to understand critical components of the T cell immune response in normal and neoplastic cells. When T-cells are activated by antigen or mitogenic lectin, both IL-2 and IL-2 receptor expression are induced. IL-2 and IL-2 receptors control the magnitude and duration of the T-cell immune response based on the amount of IL-2 produced, the levels of receptors expressed, and the time course of each of these events. Whereas low levels of intermediate affinity IL-2 receptors are expressed on resting cells, following antigen stimulation, expression of both high and low affinity IL-2 receptors is potently induced. At least three chains of the IL-2 receptor are now known to exist, namely IL-2Rα, IL-2Rβ, and IL-2Rγ. Other proteins may additionally contribute to a multichain receptor complex. In the past year, major advances have been made in a number of areas. The lab has made significant progress in studies utilizing the expression of the IL-2Rα and β chain cDNAs in 32D myeloid progenitor cells to elucidate features of IL-2 mediated signal transduction. It was shown that IL-2 can induce signals overlapping but distinct from IL-3 in IL-2Rβ transfected 32D cells capable of responding to both IL-2 and IL-3. Strikingly, IL-2, but not IL-3 potently induces IL-2Rα mRNA. Therefore, IL-2Rβ is not merely coupling into an IL-3 signaling pathway. Using two tyrosine kinase inhibitors, herbimycin A and genistein, it was shown that tyrosine kinases are critically involved for proliferation in 32D-β cells. Although other labs have suggested an essential role for p56*lck* (a *src* family kinase) in IL-2 signaling, it is clear that this tyrosine kinase is not obligately required in all cells in order to transduce an IL-2 mediated signal since p56*lck* is not expressed in 32D cells. It is possible, however, that different *src* family kinases may be capable of substituting for p56*lck*. Further, it was discovered that tyrosine kinases may be involved in the prevention of apoptosis (programmed cell death). Multiple IL-2Rβ mutants have been prepared for use in studies

intended to further elucidate the mechanisms of IL-2 signaling. It was also demonstrated for the first time that human neutrophils express IL-2 receptors and respond to IL-2. Their response allows cytotoxicity towards tumor targets and the killing of Candida albicans, a human pathogen. Thus, important progress has been made in elucidating both the mechanism of IL-2 signaling and in identifying a completely new physiological role for IL-2, a feature that could have important implications in IL-2 based immunotherapy.

Interleukin-2 receptor -- molecular regulation

As mentioned above, when T-cells are activated by antigen or mitogen lectin, both IL-2 and IL-2 receptor expression are induced. Whereas a low level of intermediate affinity IL-2 receptors are expressed on resting cells, following antigen stimulation, both high and low affinity IL-2 receptor expression is potently induced. This results at least in part from increased expression of both IL-2Rα and IL-2Rβ. cDNA encoding these proteins have previously been identified, and our lab was the first to analyze the promoters of each of these genes. In the past year, the lab has made major new advances. A new enhancer in IL-2Rα was identified. Previously, individuals in the laboratory had delineated an enhancer located between -299 and -228 relative to the more downstream of two major transcription initiation sites. This enhancer spans the IL-2Rα κB site and CArG box and exhibits potent inducibility. The new enhancer is located downstream of the first one and at least one transcription factor important for its activity has been identified. The discovery of this new κB independent enhancer may help to explain the activity of the IL-2Rα promoter in situations where NF-κB is not contributing.

In the past year, the first deletional analysis of the IL-2Rβ promoter has been performed and the critical cis-acting elements in the promoter have been delineated. In addition, two distinct regions with enhancer activity have been discovered. Both of these enhancers exhibit basal enhancer activity, and both are inducible to higher level expression in response to phorbol ester, an activator of protein kinase C.

Finally, the group has studied one transcription factor (NF-κB), which serves a pleiotropic role as a transcriptional activator of many genes, and which is critical for IL-2Rα expression in HTLV-I transformed cells. HTLV-I is a virus which causes adult T-cell leukemia, an agressive leukemia prevalent in Japan, the Caribbean basin, parts of Africa, and the Southeastern United States. A region of NF-κB p50 and p65 which is critical for DNA binding has been delineated. This region contains an interesting motif spanning a cysteine which is conserved in all κB binding proteins. In NF-κB family proteins, this cysteine mediates binding sensitivity to oxidation/reduction. Such knowledge may be critical in eventually designing therapeutic agents to act as agonists or antagonists of κB regulated genes. These findings therefore are important not only to an understanding of IL-2 receptor gene regulation but also extend to the regulation of other T-cell activation genes.

Studies related to the Act-2 cytokine:

In 1988, the group discovered Act-2 as a gene which was expressed in activated but not resting T cells. It was found to encode a secreted protein of 69 amino acids in length. Act-2 is a member of a family of small secreted proteins, many members of which have

inflammatory or chemotactic activities. This family can be divided into two subfamilies based on whether the first two of four conserved cysteines are adjacent (CC) or separated by one amino acid (CXC). Act-2 is a member of the CC subfamily. Synthesis and secretion of this cytokine are rapidly induced in T cells, B cells, and monocytes following stimulation with antigen or mitogen. In the last year, virtually all studies of Act-2 were collaborative with other laboratories. Since the last annual report, the group has published the genomic organization and chromosomal localization of this gene, and the identification of the region of the promoter which regulates expression in response to mitogens, phorbol esters and the transactivator gene of HTLV-I. During the year, it has been firmly established that the Act-2 protein has activity as a bone marrow stem cell inhibitor *in vitro*. Having previously expressed Act-2 protein in a baculovirus expression system, more than 15 milligrams of purified Act-2 protein has been prepared. Analysis of this protein by one dimensional NMR indicates that it is of high quality and suggests that it is possible to produce sufficient protein (including ^{15}N and ^{13}C labeled protein) to undertake structural analysis by multidimensional NMR. Such information is vital to eventual drug agonist/antagonist development as well as being scientifically important since no structural information is yet available on any members of the CC subfamily. Pilot studies are being performed to evaluate the feasibility of cDNA cloning of the Act-2 receptor.

ANNUAL REPORT OF THE CARDIOLOGY BRANCH
National Heart, Lung, and Blood Institute
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The experimental interests of the Cardiology Branch have evolved over the past 2 years as a result or our developing a section on molecular and cell biology, maintaining our collaborations in the area of molecular genetics, and continuing to integrate these basic disciplines with our longstanding strengths in clinical and basic physiologic investigations.

ANGIOGENESIS AND ENHANCEMENT OF CORONARY COLLATERAL DEVELOPMENT

CAD pts who develop ischemic symptoms refractory to pharmacologic therapy and who are not candidates for initial or repeat bypass surgery, pose a major therapeutic problem. Several years ago we postulated that one approach to this dilemma would be to devise a means of enhancing collateral growth, and thereby collateral flow, to ischemic regions of the myocardium.

Strategies to enhance coronary collateral development

Animal studies: In recent years we developed several canine models of myocardial ischemia to test possible approaches that might enhance collateral development. In one major study we found that heparin increased the proportion of maximal collateral flow contributed by an extra-cardiac artery implanted into the ischemic territory. Although heparin alone is not angiogenic, it potentiates the mitogenic activity of aFGF and of bFGF. Thus, ischemia might release stored FGF's and exposure to heparin might potentiate their activity, thereby increasing their capacity to promote angiogenesis.

Because of the angiogenic potential of bFGF, we previously had infused this polypeptide into the distal circumflex coronary artery (CFX) of dogs in which the CFX was subjected to gradual occlusion. We found that bFGF, admin- istered daily over 4 weeks, increased collateral flow by over 40%. This year, we found that bFGF, given *systemically* to dogs, enhanced collateral flow to a similar extent. A long range study is in progress in which bFGF is adminis- tered for varying periods of time to determine: 1) whether longer administra- tion will further increase collateral flow, and 2) whether the improved coll- ateral flow that occurs after 4 wks will persist after discontinuation of FGF. This year we also completed our investigation of the effects of vascular endo- thelial growth factor (VEGF). Unlike FGF, this growth factor is relatively specific for endothelial cells. We found the angiogenic effects comparable to those of FGF, demonstrating that stimulation of endothelial cells is sufficient for an angiogenic response. Because of its very specific activity, these results suggest that VEGF may be superior to FGF in a clinical setting.
Human studies: Our demonstration that heparin enhances collateral growth in dogs with myocardial ischemia led us to initiate a double blind, placebo-controlled study in which we examined the relative effects on collateral growth of exercise and 4 weeks of s.c. administration of a low molecular weight heparin fragment (HF), vs placebo. HF has less anticoagulant activity than whole heparin, but its capacity to enhance the angiogenic effect of FGF's is preserved. The results of this study suggest that the combination of HF treatment and exercise conditioning is angiogenic in CAD pts.

VASCULAR BIOLOGY AND CORONARY RESTENOSIS

Molecular and Cellular Biology

Vascular response to injury: Increasing evidence implicates the smooth muscle cell (SMC) in the process of atherogenesis. Specifically, it appears that SMCs are activated in

response to injury, causing their proliferation and migration from the media to the intima. A similar but markedly accelerated process causes restenosis following coronary angioplasty in 25-50% of pts weeks or months later. Several approaches to reduce restenosis incidence have been attempted but, so far, to no avail.

Rapidly proliferating cells express high levels of receptors, growth factors and proto-oncogenes that normally are absent or present at lower density in quiescent cells. We hypothesized that such changes may allow specific targeting of therapy to rapidly proliferating SMC; ie, those SMC destined to form the neointima and thereby to cause restenosis. We have undertaken two approaches to inhibit SMC proliferation by *molecular targeting*: 1)the development of recombinant chimeric toxins targeted to cell surface receptors, and 2)the use of antisense technology to inhibit the expression of specific genes. Our research goals are to develop interventions that might prevent restenosis and, in the process, to learn more about the cellular and molecular mechanisms responsible for restenosis.

Application of recombinant DNA technology to inhibit SMC proliferation following vascular injury: We utilized recombinant DNA technology to target and kill rapidly proliferating SMC. *Pseudomonas exotoxin* (PE) is a potent toxin that kills most cells; its toxicity is lost when its cell recognition domain is mutated or deleted. In collaboration with Dr. Ira Pastan, NCI, we ligated the gene fragment encoding TGFα, and that encoding aFGF to the PE gene that had a mutated binding domain; this fusion gene was expressed, producing a chimeric toxin targeted to the EGF or FGF receptor. In vitro, both of these chimeric toxins preferentially targeted and killed rapidly proliferating, as opposed to quiescent SMC.

Model of vascular injury: These encouraging results led us to develop models of vascular injury. In one model, which employs a mechanically induced injury to the central artery of the rabbit ear, we mapped the course and timing of events following vascular injury. Initially, SMC's located in the media proliferate. These cells then migrate to the subintima through perfor- ations in the internal elastic membrane, forming a neointima. They then continue to divide such that by 2 weeks the neointima has expanded consider- ably, compromising the vascular lumen. By 5 weeks, the neointima lesion has stabilized and there is no further evidence of SMC proliferation. Such a sequence of events suggests that a therapeutic window exists to inhibit SMC proliferation and migration. We therefore studied the in vivo efficacy of the chimeric toxins to inhibit SMC proliferation. Our results to date are nega- tive. However, additional in vivo studies are planned to test our hypothesis. *Antisense strategies to inhibit SMC proliferation:* We are determining whether specific genes play a role in SMC proliferation by inhibiting their expression with antisense oligodeoxynucleotides (ODN's) targeted to their mRNAs. Proliferating cell nuclear antigen (PCNA) is a nuclear protein expressed at high levels only when cells are rapidly proliferating, and is a prerequisite for DNA replication. c-myc is an immediate early response gene whose protein product probably participates in numerous signaling trans- duction pathways, including those modulating cell division. We tested several 15-18 mer antisense ODNs targeted to PCNA mRNA, or to c-myc mRNA. Antisense ODNs to both PCNA and to c-myc inhibited expression of their gene's protein products, and inhibited SMC proliferation by 50%; interestingly, antisense ODNs to c-myc also inhibited SMC migration by 90%. These results provide a major impetus for in vivo studies to determine efficacy of antisense strategies in the prevention of coronary restenosis.

Effect of retinoblastoma gene product on SMC proliferation: The role of the retinoblastoma gene product (pRb) as a critical controlling element for inhibiting cell proliferation was first suggested by the demonstration that mutations in the retinoblastoma gene (*Rb*) are frequently associated with several specific tumors, and that in gene transfer studies its overexpression in transformed cells suppresses cell growth. Additional evidence supporting such a role are the observations that its state of phosphorylation is altered with the cell cycle. We are studying the influence of suppressor genes, like *Rb*, on SMC proliferation. In collaboration with Dr. Bruce Howard, we are using a novel system that allows sensitive assessment of the effects of transfected suppressor gene activity on cell proliferation. Rat SMCs were transiently transfected to express wild type SV40 T antigen (Tag) or a mutant. This multifunctional protein binds pRb and inhibits its suppressor activity, whereas the mutated Tag cannot bind pRb. Thus, if pRb normally inhibits SMC proliferation, its binding by Tag should stimulate quiescent SMCs to proliferate. Our results suggest that the product of the retinoblastoma gene is indeed expressed in SMC in vitro, and this product seems to exert a suppressor function in the maintenance of SMC quiescence.

HYPERTROPHIC CARDIOMYOPATHY (HCM)

Genetic and molecular abnormalities (These studies were performed in collaboration with Dr. Neal Epstein, Clinical Hematology Branch.)

Genotyping: When linkage to the beta myosin heavy chain gene locus on chromosome 14 was reported (Seidman C et al.), we were able to confirm our previous report that there is non-allelic genetic heterogeneity in HCM and that βMHC gene is responsible for the disease in only a third to half of HCM kindreds. Sequencing of the βMHC gene in these kindreds has so far yielded 7 missense mutations resulting in single amino acid changes in the head or head-rod junction region of the βMHC molecule in 8 HCM kindreds.

Correlation of genotype and phenotype: Over 230 detailed family trees of more than 2000 pts have been collected and DNA samples obtained from family members. HCM kindreds have been categorized into myosin and non-myosin associated HCM. The myosin associated HCM kindreds have been further sub- divided according to distinct mutations. We have found that the various muta- tions are associated with different clinical characteristics and prognoses. Thus, the $908^{Leu \rightarrow Val}$ amino acid residue mutation (kindred 2755) is associated with a 64% penetrance in adults (by echo criteria), late onset of disease, and low sudden death incidence. By contrast, the $403^{Arg \rightarrow Gln}$ amino acid residue mutation (kindred 2002) is associated with 100% penetrance in adults, early disease onset, and high sudden death incidence. The availability of molecular markers has allowed preclinical identification of children who have inherited the disease allele. We have also identified two unrelated infants with syncopal episodes each of whom was identified as carrying the mutant gene; in each the spell was arrhythmic in origin. These two cases fit the description of "near miss for sudden infant death syndrome (near-SIDS)" event. Thus, it is possible that some instances of SIDS are due to arrhythmias occurring in children with HCM but without its typical cardiac morphologic appearances.

Expression of the βMHC gene and the pathophysiology of the protein: The βMHC gene product is the dominant heavy chain in human ventricular myosin. A myosin heavy chain of the same size is present in slow muscle fibers of human skeletal muscle. By virtue of the 908 mutation and a silent mutation which characterizes the mutant allele in kindred 2755, we have shown the same mutant message linked to the disease phenotype

is also expressed in skeletal muscle. On this basis, in collaboration with Dr. James Sellers and Dr. Giovanni Cuda (Lab of Molecular Cardiology), we showed that in vitro motility activity of antibody purified beta myosin obtained from soleus muscle of pts with the $403^{Arg \rightarrow Gln}$ and the $908^{Leu \rightarrow Val}$ amino acid residue mutations is strikingly abnormal. In collaboration with Dr. Sergey Malinchik, Dr. Robert Horowits and Dr. Richard Podolsky (NIAMD), we showed that the active force generated by chemically skinned single muscle fibers from pts with $403^{Arg \rightarrow Gln}$ mutation is significantly less than normal controls and pts with the $908^{Leu \rightarrow Val}$ mutation. In collaboration with Dr. Marinos Dalakas (NINDS), we showed that soleus muscle biopsies of HCM pts from 5 kindreds with mutations in the βMHC gene show the presence of skeletal myopathy under light microscopy. The majority of pts with 3 of the βMHC gene mutations also show signs of a rare muscle disorder called central core disease (CCD), in which type I fibers predomin- ate, many of which are missing mitochondria centrally. Biopsy samples from HCM pts with non-myosin associated HCM also show a mild skeletal muscle myo- pathy, but without CCD characteristics. Thus, familial HCM often appears to be a disorder of striated muscle in which there is a predominance of cardiac involvement.

Clinical Studies

Sudden cardiac death and syncope: We have identified, by utilizing non-invasive (exercise stress testing for detection of myocardial ischemia) and invasive (cardiac catheterization and electrophysiologic) studies, several specific mechanisms that could be responsible for sudden death in HCM. Therapy directed at these abnormalities appeared to result in improved prognosis. This study also demonstrated the value of ventricular tachycardia induced at electrophysiologic study in predicting subsequent cardiac events.

Pacemaker therapy as alternative to cardiac surgery in pts with obstructive HCM: We have shown that dual chamber pacing is effective in relieving drug-refractory symptoms and reducing LV outflow obstruction. The advantage of this therapy over traditional LV myectomy or mitral valve replacement is its low cost and lower mortality and morbidity. An important finding has been that chronic pacing alters electrical and hemodynamic cardiac properties that are evident even when pacing is discontinued. We intend to investigate the molecular basis of this further.

Radiofrequency (RF) ablation of the atrioventricular (AV) node in HCM pts with drug-refractory atrial fibrillation: About 15% of HCM pts develop atrial fibrillation. This often results in severe symptoms and hemodynamic deterioration, and may be difficult to treat with drugs. Preliminary results in about 20 HCM pts show that RF catheter ablation of the AV node and insertion of a rate-responsive ventricular pacemaker improves cardiac output, and reduces pulmonary hypertension and outflow tract gradient. Need for drug therapy is reduced and there is marked improvement of pts' functional class status.

THE CORONARY MICROCIRCULATION IN ISCHEMIC HEART DISEASE

Angina occurring in the absence of coronary artery disease or vasospasm of large coronary arteries has been a diagnostic dilemma since the advent of coronary angiography. Although noncardiac causes of pain undoubtedly account for symptoms in a subset of these pts, we have shown that the coronary microcirculation may be dysfunctional in many.

Morphologic study of hearts of pts with HCM studied at necropsy has demonstrated abnormal intramural small arteries. More recent studies have demonstrated evidence

for regional and subendocardial ischemia during stress, associated with metabolic evidence for myocardial ischemia. Although the pathophysiology of ischemia in HCM is undoubtedly complex, abnormal small arteries are likely to contribute to ischemia in this population. We have studied coronary flow reserve during stress in hypertensive pts and mild LV hypertrophy, and in pts with dilated cardiomyopathy; these have shown limited flow responses to stress, and constrictor responses of the microcirculation to ergonovine. We have also studied pts without hypertrophy (primary or secondary) who have angina-like symptoms in the absence of coronary artery disease, and who commonly have abnormal exercise stress tests by noninvasive nuclear techniques. Such pts commonly have limited coronary flow responses to stress and to pharmacologic vasodilators such as adenosine, with constrictor responses of the coronary microcirculation to ergonovine. In contrast, pts with no evidence of inducible myocardial ischemia by stress nuclear studies infrequently show such constrictor responses to ergonovine. These observations suggest that a dynamic abnormality of the coronary microcirculation may cause low flow responses to stress, resulting in myocardial ischemia.

We have also initiated studies to elucidate the pathogenesis of the microvascular dysfunction. A subset of pts with coronary microvascular dysfunction appear to have abnormal endothelial function (see below). However, the etiology of microvascular dysfunction is likely to be multifact- orial; indeed, some pts with coronary microvascular dysfunction also have abnormal smooth muscle function in other vascular and nonvascular beds, in- cluding the esophagus and bronchial airways. This observation suggests that a subset of pts may have a generalized disorder of smooth muscle function.

Although the prognosis for pts with chest pain syndromes despite angiographically normal coronary arteries has previously been demonstrated to be good, some of our pts over time have demonstrated substantial decreases in resting LV function. Of interest, the subgroup at highest risk for deterioration in LV function is comprised of pts who had abnormal nuclear stress studies during initial evaluation. This may indicate that inducible myocardial ischemia can cause progressive injury and scarring (particularly notable in biopsies performed in 9 such pts to date). Alternatively, the abnormal nuclear studies may indicate an occult cardiomyopathic process, in the absence of ischemia, with progressive deterioration in LV function over time. Future studies will focus on the etiology of deterioration in LV function in this subset, now that a high risk hemodynamic marker has been identified.

Further studies have shown that exaggerated intracardiac (and esophageal) sensitivity accounts for symptoms in a majority of pts with chest pain despite normal coronary angiograms, regardless of the presence or absence of underlying microvascular dysfunction. A pilot study performed in our Branch showed that drugs effective in neuropathic pain syndromes are also effective in this setting, and a double-blind, randomized, placebo-controlled study employing such agents is currently in progress.

ENDOTHELIAL FUNCTION OF THE MICROVESSELS: ITS CONTRIBUTION TO CORONARY VASCULAR TONE, PERIPHERAL VASCULAR TONE, AND PLATELET FUNCTION IN MAN AND ITS CONTRIBUTION TO MICROVASCULAR DYSFUNCTION

The endothelium of blood vessels secretes substances that dilate vascular smooth muscle and inhibit platelet aggregation. These factors are known as endothelium-derived relaxing factors (EDRFs). This year we have continued our studies designed to elucidate the normal mechanisms by which the endothelium modulates coronary and systemic vascular tone and influences platelet function, and how aberrations of its normal function

may influence and contribute to the development of various disease states.

Endothelial abnormality in microvascular angina (MVA): About 40% of pts with chest pain and normal coronary arteries (syndrome X) have reduced microvascular vasodilation in response to atrial pacing (MVA). We found that these pts also have reduced endothelium dependent dilation (as assessed with acetylcholine). The magnitude of endothelial dysfunction correlated with the magnitude of the impaired vasodilator response to atrial pacing. Similar results were found in pts with hypertension who have chest pain and normal coronary arteries. Thus, endothelial dysfunction of the coronary microvasculature appears to account for the reduced vasodilator reserve of the coronary microvasculature in some pts with MVA, and in some with hypertension.

Role of endothelium derived nitric oxide (NO) in exercise-induced vasodilation: EDRF (NO) is tonically released from blood vessels and produces smooth muscle vasodilation. Whether it has a physiologic function during exercise-induced vasodilation, however, was unknown. We found that inhibition of EDRF/NO release with L-NMMA (which impairs synthesis of NO) reduces exercise-induced forearm vasodilation, indicating that the endothelium does contribute to exercise-induced vasodilation through release of EDRF/NO.

Contribution of EDRF to platelet aggregability in man: NO is a potent inhibitor of platelet aggregation (PA); therefore, lumenal release of NO by endothelial cells may normally modulate platelet function. We hypothesized that if endothelium is dysfunctional, as it is in CAD, its role in inhibiting PA may be compromised, a defect that may contribute to the precipitation of acute ischemic syndromes. We investigated the effect of stimulation of EDRF release with acetylcholine on PA in normal subjects and in those with atherosclerosis. Acetylcholine infusion (iv) inhibited PA by 73% in normals, but by only 31% in pts with atherosclerosis. Thus, endogenously produced EDRF inhibits platelet function in vivo, and its effect is blunted in pts with dysfunctional endothelium due to atherosclerosis.

Platelet activation in coronary artery disease: Platelet activation (PAc) and resulting PA may contribute to precipitation of acute, and exacerbation of chronic, ischemic syndromes. We previously found that in pts with CAD PAc occurred across the coronary vascular bed during atrial pacing and mental stress. Atrial pacing was repeated after iv infusions of either nitroglycerin or sodium nitroprusside, which increase intracellular levels of cyclic GMP. Both agents inhibited tachycardia-induced PAc in the coronary sinus. Aspirin, which inhibits the cyclo-oxygenase system, produced similar effects. Thus, tachycardia or exercise-induced PAc across the atherosclerotic coronary circulation can be inhibited by nitrovasodilators or aspirin.

Endothelial function in essential hypertension: We previously found that pts with hypertension have an abnormal response to endothelium-dependent vasodilators, an abnormality not reversed by conventional antihypertensive treatment. However, because several factors are released by the endothelium, these findings do not identify the specific factors involved. We hypothesized that a reduced synthesis or release of endothelium-derived NO may account for this abnormality. We therefore investigated the forearm response to an inhibitor of the synthesis of nitric oxide by endothelial cells (N^G monomethyl L-arginine [LNMMA], an analogue of L-arginine) and to L-arginine, the natural precursor of NO. Inhibition of the synthesis of NO produced significant vasoconstriction in normal humans, but this response was blunted in hypertensive pts. In addition, inhibition of the synthesis of NO by LNMMA in normal humans produced a significant blunting of the response to acetylcholine; however, such a response was not observed in hypertensive pts. Increased availability of L-arginine, the natural precursor for NO synthesis, significantly augmented the response to acetylcholine in normal humans. However, no signifi-

cant change was observed in the response to acetylcholine in pts with essential hypertension. These findings indicate that hypertensive pts have a decreased release of NO both basally and during stimulation with endothelium-dependent vasodilators, and that this defect in the endothelium-derived NO system is not due to decreased availability of its natural substrate.

Endothelial function in pts with hypercholesterolemia: Previous studies have shown that experimental animals and humans with hypercholesterolemia have impaired response to endothelium-dependent vasodilators. To study the role of endothelium-derived NO system in this abnormality, we studied the response of endothelium-dependent and endothelium-independent vasodilators in hypercholesterolemic pts and normal controls before and after inhibition of the synthesis of NO by an arginine analog (L-NMMA). The results showed that pts with hypercholesterolemia have impaired endothelium-dependent vascular relaxation. Inhibition of NO synthesis produced a similar vasoconstrictor response in normals and in hypercholesterolemic pts. However, in contrast to normals, the response to acetylcholine in hypercholesterolemic pts was not significantly modified by inhibition of NO synthesis. These findings suggest that hypercholesterolemic pts have a normal release of endothelium-derived NO during resting conditions but an impaired release during stimulation with endothelium-dependent vasodilators. This abnormality may account for the impaired endothelium-mediated vascular relaxation observed in these pts.

CORONARY ARTERY DISEASE

Identification of viable myocardium in pts with LV dysfunction: The differentiation of viable from nonviable myocardium in pts with coronary artery disease and LV dysfunction is of great clinical relevance. Until recently, determining whether impaired regional or global LV function at rest was a potentially reversible process could be made only retrospectively, after the pt had undergone coronary artery angioplasty or bypass surgery. Our studies demonstrated that viable but dysfunctional myocardium in such pts can be identified prospectively by thallium-201 scintigraphy, using an exercise-redistribution-reinjection protocol. That myocardial regions identified by thallium reinjection method represent viable myocardium is supported by: 1) improvement in both regional perfusion and regional wall motion following revascularization, 2) preserved metabolic activity by PET, 3) preserved regional systolic wall thickening by gated nuclear magnetic resonance imaging. Furthermore, in regions with persistent thallium defects despite reinjection, the severity of reduction in thallium activity (mild-to-moderate versus severe) can further differentiate viable from nonviable myocardium.

However, if the clinical question being addressed is whether a non-contractile region is viable (scar vs ischemia), then it may be more reasonable to perform rest-redistribution imaging rather than stress-redistribution-reinjection imaging. Recent investigations have demonstrated that although rest-redistribution thallium imaging identifies viable myocardium in the majority of reversible regions, it may still underestimate viable myocardium. The greater accuracy achieved by stress-redistribution-reinjection imaging when compared with rest-redistribution studies may arise from the greater initial flow heterogeneity created during exercise, and hence the greater potential for demonstrating reversal on subsequent images in regions with reduced perfusion at rest.

Data regarding the use of technetium-99m sestamibi for identifying viable myocardium are preliminary. As sestamibi tracks with regional myocardial blood flow but does not redistribute appreciably, this agent may not differentiate viable myocardium with chronically reduced perfusion from fibrotic myocardium. Preliminary data from our laboratory

indicate that rest and stress sestamibi images will incorrectly identify 36% of myocardial regions as being irreversibly impaired and nonviable when compared to both thallium redistribution-reinjection and PET. However, if a late redistribution image is acquired following the rest sestamibi injection, or if the severity of reduction in sestamibi activity within irreversible defects is considered, the identification of reversible and viable myocardium can be greatly enhanced with sestamibi.

Annual Report of the Clinical Hematology Branch
National Heart, Lung and Blood Institute
October 1, 1991 to September 30, 1992

The research of this Branch is directed toward understanding the underlying causes and developing effective treatment for major hematological disorders, including thalassemia, sickle cell anemia, and various syndromes of bone marrow failure and myelodysplasia. The scope of our work is broad and includes basic study of the molecular mechanisms of gene regulation and extends to applied clinical trials of specific therapeutic agents. Modern methods of molecular and cell biology, including recombinant DNA technology, are utilized in this comprehensive approach to disease mechanisms and therapy. The following are several highlights of progress made in our research during the past year.

MOLECULAR BIOLOGY SECTION

Gene Insertion into Hematopoietic Stem Cells

Gene replacement as therapy for genetic and acquired bone marrow disorders has become a realistic research goal. Our efforts have focused on the biology of hematopoietic stem cells and means to manipulate these cells in vitro to achieve gene insertion. Most stem cells are quiescent and in the G_0 phase of the cell cycle. Retroviral mediated gene transfer requires cell division. In the murine model, we have systematically investigated the ability of various growth factors to promote amplification of stem cells in vitro. The combination of interleukin-3, interleukin-6 and stem cell factor (SCF) seem optimal for this purpose. SCF has been shown to effect mobilization and amplification of stem cell numbers in murine tissues. Currently, we are investigating the ability of various autologous, allogeneic and genetically engineered stromal cell lines to support proliferation of highly purified murine stem cells.

Insights acquired from studies in the murine model are applied in an autologous bone marrow transplantation model in non-human primates. Early hematopoietic stem cells are purified from rhesus bone marrow by positive immunoselection, cultured in vitro with recombinant growth factors in the presence of retroviral vectors and transplanted after the animal has received total body irradiation to ablate remaining bone marrow cells. During the past year, we observed a rapidly progressive malignant lymphoma in three animals transplanted with cells exposed to a vector preparation contaminated with replication-competent retrovirus. Multiple copies of the replication-competent viral genome were present in the tumor cells suggesting insertional activation of proto-oncogenes and inactivation of tumor supressor genes as the mechanism of oncogenesis. These observations establish that murine retroviruses are pathogenic in primates and underscore the critical

importance of avoiding replication-competent virus contamination in vector preparations used for clinical studies. We have succeeded in achieving retroviral mediated gene transfer into repopulating hematopoietic stem cell, using helper-free vector preparations. The gene for adenosine deaminase has been transferred and expressed in rhesus cells. We have also inserted the human multi-drug resistance (MDR) gene into repopulating primate stem cells in pre-clinical studies designed to investigate the potential of using this gene to create drug resistant bone marrow in patients being treated for neoplastic disorders.

During the past year we have begun a program of autologous bone marrow transplantation for patients with multiple myeloma and chronic myelogenous leukemia. Pre-clinical studies in the non-human primate model and with human cells in vitro have provided data supporting development of stem cell retroviral marking protocols in these disorders. The protocols have now been approved by the Institute Review Board and the Recombinant DNA Advisory Committee. We anticipate that attempts at gene transfer into human bone marrow stem cells in the context of autologous bone marrow transplantation will begin within the next year.

Regulation of Hemoglobin Switching

Patients with either severe beta-thalassemia or sickle cell anemia could benefit from increased production of fetal hemoglobin. At the gene level, the switch reflexes turn-off of the γ-globin genes and turn-on of the β-globin genes. If both beta genes are defective, the switch leads to the onset of hematological disease. The β and γ genes are part of a multi-gene cluster on chromosome 11 that extends over 60,000 base pairs of DNA. Human globin genes exhibit tissue and developmental stage specificity. Gene expression increases dramatically during progression from pro-erythroblasts to the latter stages of erythropoiesis. Regulation of the globin genes is achieved by factors that bind DNA with sequence specificity (trans-acting factors). These proteins bind to cis-acting elements within and flanking the individual genes in modulating gene expression.

Studies in prior years have established that various pharmacological agents are able to augment HbF synthesis in patients with sickle cell anemia and thalassemia. However, the real solution to therapeutic hemoglobin switching will come from a thorough knowledge of the regulatory mechanisms that control gene expression. Accordingly, most of our efforts have focused on identifying specific cis-active elements involved in switching. These include a powerful enhancer within the locus control region upstream from the cluster, a stage selector element within the γ-globin gene promoter and a regulatory element downstream from the γ-globin gene. Specific proteins that interact with each of these elements have been identified by a combination of functional and DNA binding assays. Our work in the past year has focused on purifying these proteins and obtaining molecular clones of their coding sequences. Two proteins have been purified and purification

of a third is nearing completion. In future studies, we will concentrate on the functional significance of these proteins in modulating switching and hope ultimately to devise vectors that can be used to overexpress each protein in bone marrow cells with resulting resumption of fetal hemoglobin synthesis.

Molecular Defect in Hypertrophic Cardiomyopathy

Hypertrophic cardiomyopathy is a dominantly inherited genetic disease of the heart, manifested by left ventricular outflow obstruction, cardiac failure, arrhythmia and/or sudden death. Investigators in the Clinical Hematology Branch in collaboration with researchers in several other Branches at the NIH have undertaken a genetic, molecular biological and functional approach to this disorder. Seven differents mutations in the portion of the β myosin heavy chain gene that encodes for the head or rod region have been identified in separate families. Pre-symptomatic diagnosis can now be accomplished in these families and mutation specific disease evolution and prognosis is being defined. We have shown that the β myosin heavy chain gene is expressed in skeletal muscle and that mutations in this gene lead to a non-progressive, largely asymptomatic skeletal myopathy called central core disease. The functional significance of the β myosin heavy chain gene mutations has been proven by demonstration that purified myosin mutants exhibit abnormal motility in vitro.

CELL BIOLOGY SECTION

The major research and clinical interests of the Cell Biology Section of the Clinical Hematology Branch are directed toward the pathogenesis, pathophysiology, and effective treatment of bone marrow failure states. The hematology clinical service, now located on 8 East, admits a large number of patients with bone marrow failure syndromes, especially aplastic anemia, myelodysplasia, anemia, leucopenia, and undiagnosed pancytopenia. In addition, the Section serves as a reference center for physicians with questions concerning both the management of patients with aplastic anemia and for research studies, especially of parvovirus infection. Finally, the spectrum of clinical disease treated by our service has expanded with the introduction of autologous bone marrow transplantation for chronic myelogenous leukemia and multiple myeloma and the establishment of an allogeneic bone marrow transplantation unit. Physical renovation of the 2 West ward is underway for a modern, four-bed unit, and a senior bone marrow transplantation expert has been recruited to head this unit.

B19 Parvovirus

B19 parvovirus was discovered in 1975 and has been associated with a number of human diseases. The virus is the etiologic agent

of fifth disease, a common childhood rash illness which presents as an arthralgia/arthritis syndrome in adults; some cases of hydrops fetalis; transient aplastic crisis of hemolytic disease, especially sickle cell disease; pure red cell aplasia in immunosuppressed hosts; and congenital red cell aplasia in "cured" hydrops. Clinical assays for parvovirus-specific antibody and for viral genome have not been widely available, so it is likely that the clinical spectrum of the disease will be expanded. We have previously demonstrated that the virus is pathogenic because it directly infects erythroid progenitor cells in the bone marrow and that the immune response, especially production of neutralizing antibodies, restricts and terminates parvovirus infection in infected humans.

This year's major findings have come from tissue culture and recombinant molecular biology experiments. These studies have focused on the description of a human cell line that supports B19 parvovirus propagation; vaccine development with a baculovirus system for protein expression; adaptation of baculovirus-generated capsids for protein presentation; and studies of a putative B19 parvovirus receptor.

We have previously shown that the megakaryoblastoid human cell line called UT7 supports parvovirus propagation. The virus can replicate and produce new viral proteins and infectious particles, but over time both DNA replication and protein production decline. Current studies show that the virus is latent in these cells and that virus replication can be induced by cell synchronization. Furthermore, careful analysis of the kinetics of virus in freshly infected cells has shown both early and late gene expression events, with nonstructural protein RNA expressed within hours of infection followed about 12 hours later by structural protein RNA and polypeptide synthesis and DNA replication. Taken together, and combined with clinical observations, these results suggest that human cells can be abortively infected by human parvovirus and gene expression limited to the cytotoxic nonstructural protein.

We have a Collaborative Research and Development Agreement with MedImmune Inc., and with them have demonstrated that recombinant parvovirus capsids produced in a baculovirus system can elicit neutralizing antibodies in different animal species. Of great importance is the contribution of the minor capsid protein gene called VP1. Normally parvovirus virions contain only about 5% VP1. However, we have shown that VP1 is absolutely required for a neutralizing antibody response and, furthermore, that increases in the amount of VP1 in recombinant capsids improves the titer of neutralizing antibodies in immunized animal serum. In current studies, we have demonstrated that the unique region of VP1, 226 amino acids at the amino terminal side of the VP2 molecule, is exposed externally on the capsid. This area contains multiple neutralizing epitopes, but insertion of VP1 in the capsid also alters allosterically the predominant major capsid protein, VP2. As VP1-deficient capsids bind in hemagglutination assays similarly to virions, it is unlikely that the VP1 unique region represents

the receptor ligand. By analogy to the coronaviruses, we suspect that cleavage of the VP1 unique region may be important for internalization of the virus and decapsidation of its DNA into the cell. Careful analysis using sera produced against fusion proteins and peptides representative of the entire capsid sequence has indicated that a region particularly capable of eliciting neutralizing antibodies lies at the junction site of VP1/VP2, consistent with this hypothesis.

Because VP1 is not required for capsid assembly but is external to the capsid, we have begun to produce parvovirus capsids that function as protein platforms for foreign or heterologous amino acids sequences. The unique region of VP1 has been substituted by sequences from the gp120 envelope protein of the human immunodeficiency virus or the hemagglutinin protein of human influenza virus. Empty capsids can form containing these heterologous sequences when SF9 cells are co-infected with appropriate baculovirus recombinant viruses. These studies indicate that large, non-parvoviruses sequences can be substituted for the unique region of VP1 without perturbing capsid assembly.

Finally, preliminary studies have indicated a useful assay for a parvovirus receptor. B19 parvovirus can hemagglutinate sheep and monkey red blood cell, and we are exploiting this property to isolate the parvovirus receptor. A number of biochemical manipulations of erythrocytes has indicated that the receptor is likely to be a glycoprotein of moderate molecular weight. Furthermore, the ability of certain fusion proteins and peptides derived from the parvovirus sequence to block hemagglutination has suggested that the major spike region of the parvovirus capsid (rather than the canyon) is the binding site for virus to cells.

In the coming year, these projects will be continued. We believe that there is a high likelihood that we will be able to isolate, for the first time, a parvovirus receptor on a mammalian cell. Computer analysis of parvovirus capsids protein structure has suggested portions of the unique region of VP1 that interact with the internal surface of the capsid cylinder which may be required for presentation of the most amino terminal portions of VP1 to the surface. Site- directed immunogenesis followed by capsid assembly in baculovirus is being used to determine the importance of these sequences and the optimal structures for presentation for heterologous proteins in the recombinant system. Finally, we hope to enter human vaccine trials with recombinant B19 parvovirus in the next 1-2 years, first with normal volunteers and then in patients with sickle cell disease who are at risk for transient aplastic crisis.

Etiology of Aplastic Anemia

Aplastic anemia has clinical and laboratory features that suggest that viruses or drugs can incite pathophysiologic immunologic response. We have previously demonstrated the presence

of activated cytotoxic lymphocytes that over express gamma-interferon in the blood of many patients with this disease. As described in last year's report, the hepatitis/aplasia syndrome may be a particularly good example of the role of a virus in producing bone marrow failure, and this syndrome is not due to any known hepatitis virus. To pursue the virus, chimpanzee studies involving inoculation of sera from patients with hepatitis/aplasia and liver extracts from patients with fulminant hepatitis have been undertaken. This year's major work has been directed at the immune pathophysiology, as well as in advances in the treatment of aplastic anemia.

We have developed a sensitive gene amplification method for the detection of gamma-interferon mRNA in extracted RNA from peripheral blood and bone marrow. These experiments were undertaken because of the likelihood that local production of hematopoietic suppressor molecules by cells of the immune system is more important than detection of these molecules in the circulation, analogous to the concentration of hematopoietic growth factors in the bone marrow and their spill-over into peripheral blood. Using the polymerase chain reaction, we have detected gamma-interferon mRNA in 9/12 aplastic bone marrows obtained at the time of first presentation for therapy at the Clinical Center. Gamma-interferon has not been detected in a large number of normal bone marrows or in control samples obtained from patients who are multiply transfused or with other causes of pancytopenia. These results extend our previous experiments implicating gamma-interferon and cytotoxic lymphocytes in this form of bone marrow failure. Our studies are continuing in larger numbers of patients and will be correlated with other parameters of immune dysfunction and with the clinical course, especially response to immunosuppressive treatment of patients.

We have successfully implemented an intensive combined immunosuppressive protocol for patients with severe aplastic anemia on presentation. This treatment consists of anti-thymocyte globulin (ATG) at 40 mg/kg/day combined with cyclosporine 12-15 mg/kg/day. In approximately 30 patients treated to date, the overall response rate is approximately 68%, well above our historical response rate of 45%. These results suggest that intense immunosuppression is superior to conventional immunosuppression in patients with severe aplasia.

To be undertaken in the coming year are innovative studies of growth factors in aplastic anemia, including interleukin-3 in Diamond-Blackfan anemia, to extend our previously published result that this hematopoietin can induce long-term remissions in congenital anemia, and stem cell factor, a novel cytokine that acts on primitive hematopoietic stem cells, for patients with refractory severe aplastic anemia.

ANNUAL REPORT OF THE HYPERTENSION-ENDOCRINE BRANCH
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October I, I99I through September 30, I992

The Hypertension-Endocrine Branch pursues studies into the mechanisms of blood pressure regulation and the causes and treatment of hypertension. Our activities have concentrated in three areas: I) vasoactive substances, 2) molecular mechanisms of cardiac hypertrophy, and 3) studies of pheochromocytoma.

I. Vasoactive substances. Atrial natriuretic peptide (ANP) (99-I26) is a natriuretic peptide produced in the heart of most mammals. Recently, urodilatin (Uro) (ANP 95-I26) has been discovered and added to this family of natriuretic peptides. It has the same structure as ANP 99-I26 with four additional amino acids on the amino terminal end. These are the same amino acids found in pre-pro ANP and therefore suggest that Uro is a product of the same gene, but with alternate cleavage at the time of synthesis. One major problem with ANP is that it rapidly loses its effectiveness in situations where it is most needed. Thus, plasma and urine levels of ANP are markedly elevated, and exogenously administered ANP is less effective in animals and people with congestive heart failure. We showed in control rats that administration of either ANP or Uro resulted in dose-dependent increases in urine output and sodium excretion. In rats with congestive heart failure, administration of ANP had little effect, while administration of Uro still produced significant increases in urine flow and sodium excretion, but the absolute levels were lower than those found in control rats. There is evidence that both ANP and Uro are subject to removal from the circulation by the action of a clearance receptor. In addition, ANP is subject to degradation by a neutral endopeptidase, to which Uro is relatively resistant. When 40 mg/kg of the neutral endopeptidase inhibitor (NEPI), SQ 28063, was infused into rats with congestive heart failure, the absolute urine flow and sodium excretion were increased to the same levels as those obtained when the same dose of Uro was given to control rats. Infusion of equimolar doses of ANP plus NEPI in the control rats and rats with congestive heart failure resulted in a diminished natriuretic and diuretic response compared to those obtained after infusion of Uro plus NEPI. Thus, we have shown that Uro is more useful than ANP in the treatment of cardiac edema, and the combination of Uro plus an NEP inhibitor is even better therapy.

Since the four additional amino acids at the amino terminal end of Uro are the same ones which immediately precede ANP in its precursor molecule, it is probable that they are the product of the same gene.

However, while the concentration of ANP in the blood is high, that of Uro is low. In fact, Uro is found mainly in urine. Thus, it is probable that Uro is made in the kidney. We therefore attempted to determine the level of Uro in the kidney and the message for ANP/Uro in the kidney. We have demonstrated, for the first time, renal transcription of the gene coding for ANP and we are now trying to locate the site of this transcription in the kidney by means of in situ hybridization. We are also attempting to quantify the level of transcription to see if what we have detected by the ultrasensitive PCR reaction is really significant.

Endothelin (ET) is a powerful vasoconstrictor produced in vascular endothelium. Only very low levels are present in plasma, but we found relatively large amounts in urine. In addition, the levels of ET in blood and urine are significantly lower in hypertensive than in normotensive subjects. In rats, we showed that doses of Angiotensin II (A-II) produce significant elevations in urinary ET. When A-II was combined with the nonspecific A-II antagonist, saralasin, there was actually an additive effect on urinary ET production. Saralasin has partial agonist activity. The effect of A-II was blocked by the specific A-II antagonist, losartan. Infusion of arginine vasopressin also increased blood pressure similar to A-II, but had a much reduced effect on urinary ET. Administration of the calcium channel blocker, nifedipine, dropped blood pressure significantly, but still increased urinary ET production. However, the latter was blocked by the administration of captopril, indicating that the fall in blood pressure secondary to nifedipine caused a release of A-II which increased urinary ET production. We have recently begun to study the effects of A-II and the alpha-adrenergic agonist, phenylephrine (Phe), on urinary ET in man. In man, somewhat different results were obtained. During equipressor doses of A-II and Phe, A-II roughly doubled ET excretion, while Phe increased ET excretion seven-fold. Thus, we still have much more work to do to explain the physiological control and the role of such a potent vasoconstrictor in the kidney.

II. Cardiac hypertrophy is one of the major consequences of sustained hypertension and it can also occur in cases of volume overload. It is a major risk factor of cardiac morbidity and mortality. We sought to determine the molecular signals which transduce the increased physical pressure and hemodynamic stress into the hypertrophic response. We studied cardiac hypertrophy in several different situations, i.e., chronic administration of alpha or beta adrenergic agonists, A-II or ET, and in spontaneously hypertensive rats. We found that two distinct patterns of cardiac hypertrophy are induced by different hormonal stimuli. The first type, initiated by excessive exposure to a beta adrenergic agonist, is characterized by a rapid onset of hypertrophy, a marked increase in the

weight of the heart, reversibility of the hypertrophy after cessation of the stimulus, dependence of the response on intrinsic, as well as extrinsic, A-II levels, and dependence of the extent of the response on the age of the animal (i.e., the response was greater in younger animals). The second type of response, induced by exposure to an alpha adrenergic agent, is characterized by a slow onset of hypertrophy, a relatively small increase in heart weight, irreversibility of the hypertrophy even after cessation of the stimulus, and independence of the response to A-II levels and to the age of the animal. We are now attempting to determine how the two patterns differ in morphological and molecular aspects and to identify the specific genes, the expression of which differentiate the hypertrophic heart from the normal heart by means of Northern blotting, RNAse protection assays for known candidate genes, and by subtractive hybridization in the search for new candidate genes. So far, the cardiac hypertrophy characteristic of hypertension seems to show a combination of both patterns. Elucidation of the molecular events in cardiac hypertrophy may be helpful in the design of better agents to treat hypertension and, at the same time, prevent or reverse the development of cardiac hypertrophy.

The expression of insulin-like growth factors I and 2 and their receptors was not elevated in hypertrophy produced by either high output heart failure or in spontaneously hypertensive rats. The expression of basic fibroblast growth factor was mildly increased in these models of cardiac hypertrophy, but the elevation may only reflect an increase in the amount of connective tissue in the hypertrophied heart, rather than an increase in the effect of the growth factor.

III. We have continued our efforts in the diagnosis, localization, and treatment of pheochromocytoma. We have clearly demonstrated again the utility of our biochemical approach to the diagnosis of pheochromocytoma. This has included both basal assays of plasma catecholamines, as well as the use of glucagon as a provocative test, and clonidine as a suppression test. This eventually led to the correct diagnosis in a most difficult patient who was self-administering epinephrine in an attempt to produce a pseudo-pheochromocytoma. We also demonstrated the utility of the assay for chromogranin A in the correct diagnosis of exogenous administration of catecholamines.

There is still some debate about whether it is worthwhile to use metyrosine, the blocker of catecholamine synthesis, in the preoperative preparation of patients with pheochromocytoma. Our study of patients treated preoperatively with either Dibenzyline alone or Dibenzyline plus metyrosine showed that the combination of Dibenzyline plus metyrosine

resulted in better blood pressure control, less blood loss, and the need for less intraoperative fluid replacement than the traditional method of single-agent alpha adrenergic blockade.

We have clearly demonstrated that the severe myocardial depression that can occur after massive catecholamine release is reversible. Previously it was felt that such severe degrees of myocardial depression were uniformly fatal. We showed that the myocardium could recover in the relatively short period of 6-l0 days. This demonstrates that heroic measures are warranted to support these patients until their stunned myocardium can recover.

Molecular Disease Branch
National Heart, Lung, and Blood Institute

Cholesterol and triglycerides are carried in the blood by lipoproteins which contain proteins, designated apolipoproteins (apo) and lipids (cholesterol and triglycerides). The overall objective of the basic and clinical research program of the Molecular Disease Branch is the elucidation of the role of plasma lipoproteins in lipid transport in normal individuals and in patients with elevated plasma levels of cholesterol and triglycerides who are at risk for the development of early heart disease or other diseases including pancreatitis. A major focus of research is the analysis of the physiological role of apolipoproteins and lipoproteins in lipid transport, and the elucidation of the mechanisms involved in the regulation of lipoprotein biosynthesis, transport, and catabolism in normal subjects and patients with disorders of lipoprotein metabolism and atherosclerosis. In addition, studies are conducted to determine the molecular defects in patients with elevated blood lipids in order to establish improved methods to detect individuals at risk for early heart disease and to develop more effective drugs for the treatment of hyperlipidemic patients.

I. Gene Regulation Of Plasma Apolipoproteins and Lipolytic Enzymes

A. Apolipoproteins

The molecular mechanisms which modulate apolipoprotein and lipolytic enzyme gene expression are not well established and are important to our understanding of lipoprotein metabolism. The liver and intestine are the major sites of apolipoprotein synthesis. Lipolytic enzymes, including lipoprotein lipase, are synthesized primarily in adipose and muscle tissues. Our Branch has evaluated apolipoprotein and lipoprotein lipase gene expression at both the transcriptional and post-transcriptional levels.

Initial studies on apolipoprotein gene expression focused on apoB, the major structural apolipoprotein, on chylomicrons and VLDL. The biosynthesis of apoB has been of particular interest since the elucidation of the mechanism for the biosynthesis of the two plasma isoproteins of apoB. The two B apolipoproteins, apoB-100 and apoB-48, are synthesized from a single apoB gene present on chromosome 2 by a mechanism which introduces a stop codon in the apoB mRNA. ApoB-100, the major apolipoprotein on LDL, contains 4536 amino acids and is translated from the full length 14.1 mRNA. A second mRNA is generated by a unique editing mechanism in which the CAA codon for the amino acid glutamine residue 2253 is edited to UAA which is a stop codon and terminates the synthesis of the apolipoprotein. Translation of the edited mRNA results in synthesis of apoB-48 which contains 2152 amino acids. In man and the rat intestinal apoB mRNA is approximately 85% edited and the major apoB isoprotein secreted is apoB-48. In the human liver, apoB-100 is the primary isoprotein secreted. In contrast, in the rat, approximately 65-70% of liver apoB is edited and both the B-48 and B-100 equivalent apolipoproteins are synthesized and secreted.

A systematic analysis has been performed to establish the time course and developmental signals that determine the tissue specific expression of apoB editing. In the rat intestine, the percentage of apoB transcripts which undergo editing increases dramatically the day before birth (from ~1% to adult levels of 80%), while the liver of the rat acquires adult editing capacity during the third post-natal week (rising from ~8% to 30%). In contrast to the rat, the human small intestine acquires adult levels of apoB mRNA editing early in fetal development, rising from 10% at 10 weeks to 80% by the end of the second trimester.

ApoB editing has been proposed to play an important role in lipid metabolism. Hypothyroidism is associated with elevated plasma apoB levels and is reversed when the patient is treated with thyroxine. The molecular mechanism of T3 modulation of apoB remains to be established. Studies have been undertaken to determine the time course and developmental signals that govern the tissue specific expression of the apolipoprotein B editing process. Human liver and intestinal cell lines were transfected with a plasmid encoding the nuclear receptor.

Intestinal cells expressing receptor were treated with T3 which resulted in a six-fold increase in the expression of the apoB mRNA editing process compared to either non-transfected or non-T3 treated control cells. Initial studies have also indicated that the human liver cell line Hep G2 is capable of editing 7% of mRNA transcripts with over-expression of the T3-nuclear receptor. Co-transfection of the human growth hormone gene resulted in the synthesis and secretion of a protein consistent with apoB-48. These studies provide the first insight into the molecular mechanism whereby thyroid hormone modulates apoB gene expression and the apoB mRNA editing process.

A major goal of research on the factors which modulate apoB gene expression is the development of new potential pharmacological approaches to the reduction of apoB secretion since apoB containing LDL is a major risk factor for the development of premature cardiovascular disease (CVD). Vanadate has been shown to inhibit phosphotyrosine phosphatase and RNAase's. In the human cell line Caco-2, vanadate resulted in an 90% decrease in apoB protein production and 80% decrease in apoB mRNA levels. In addition, there was a 3 fold increase in the proportion of the apoB-48 mRNA in the vanadate treated cells. Thus, vanadate may represent a new approach to the modulation of plasma LDL levels by decreasing apoB synthesis since other agents decrease LDL levels primarily by increasing catabolism.

Several additional post-translational modifications of the apolipoproteins have been identified which may play a central role in cellular processing and secretion of the plasma apolipoproteins. Modifications of the apolipoproteins thus far identified include intracellular phosphorylation, fatty acid acylation, and glycosylation. Recent transfection studies using native apoA-I and apoA-I in which the site of phosphorylation, serine residue 201, was changed to alanine by site-directed mutagenesis established that phosphorylation was not required for biosynthesis and secretion from the cell. ApoA-I phosphorylation may therefore play a role in extracellular metabolism of apoA-I. Additional studies have shown that apoB is also phosphorylated on serine residues by protein kinase C both in vitro and in vivo. Phosphorylation increased intracellular apoB degradation indicating that phosphorylation may be an important modulator of the cellular secretion of apoB.

Evaluation of apoA-I by electrospray-mass spectrometry revealed that fatty acid acylation of plasma apoA-I is present only on proapoA-I and not mature apoA-I suggesting that acylation may be important in extra cellular apoA-I metabolism. 0-glycosylation has been established as an important modulator of the distribution of apoA-II on plasma HDL. Glycosylated apoA-II associated with the HDL3 subfraction while the non-glycosylated apoA-II was present on all HDL subfractions. These combined results indicate that a variety of post-translational modifications of the apolipoproteins may play a central role in apolipoprotein biosynthesis, secretion, and catabolism.

B. Lipoprotein Lipase

Lipoprotein lipase (LPL) is an intravascular enzyme which is attached to the capillary endothelium and is the principal enzyme involved in the hydrolysis of plasma triglycerides. A detailed knowledge of the structure and function of LPL is critical for our understanding of the biological function of this key enzyme in lipid and lipoprotein metabolism. Current studies have utilized the techniques of site-directed mutagenesis and construction of chimeric enzymes to gain insight into the functional domains of LPL. LPL, hepatic lipase (HL), and pancreatic lipase (PL) have a high degree of structural homology and form a lipase gene family. The crystalline structure of PL has been determined and serves as a structural model for the lipase gene family.

LPL contains a 22 amino acid loop defined by cys-216 and cys-239 which has been proposed to play an important role in the access of the lipid substrate to the catalytic site of the enzyme. Two amphipathic helices are present within this loop. Eight different mutants were generated in which the amphipathic properties of the loop were altered. Disruption of the amphipathic helices abolished the ability of LPL to hydrolyze the emulsified substrate, triolein, but not the monodisperse substrate, tributyrin. Substitution of the LPL loop by an equally amphipathic structure, the HL loop, preserves the hydrolytic activity against both substrates. These studies indicate that the LPL loop and its amphipathtic helices are essential for hydrolysis of emulsified,

long chained fatty acid triglycerides and provides mayor new insights into the role of the LPL loop in lipid-substrate interactions.

Chimeric enzymes have been constructed and in one of these enzymes the N-terminal 314 residues of human LPL and the C-terminal 147 amino acids of human HL have been expressed in an in vitro mammalian system. Functional characterization of this chimera has established that the site of interaction of LPL with its cofactor, apoC-II, has been localized to the N-terminal 314 residues of LPL. This region is also responsible for defining the catalytic properties of the lipase. The C-terminal domain, on the other hand, plays an important role in lipid substrate interaction and heparin binding. These combined studies provide new information on the structure and function of LPL and are a prerequisite for our analysis of mutations of LPL in clinical disorders of triglyceride metabolism as well as ultimately gene
therapy for these diseases.

II CLINICAL DISORDERS OF LIPOPROTEIN METABOLISM

Elevated blood levels of LDL and reduced levels of HDL have been well established as important risk factors for the development of premature CVD. In contrast, elevated levels of HDL have been correlated with a reduced risk of CVD and longevity. A major focus of research in the Branch is a systematic analysis of HDL metabolism in normal subjects and in individuals with both elevated and reduced levels of HDL.

A. LpA-I and LpA-I, A-III metabolism in normal subjects

Three major apolipoproteins present in HDL are apoA-I, apoA-II, and apoA-IV. Plasma HDL are heterogeneous and different lipoprotein particles within HDL have been proposed to have different physiological functions. Two important classes of lipoprotein particles within HDL are particles which contain both apoA-I and apoA-II (LpA-I,A-II), and particles which contain only apoA-I (LpA-I). LpA-I has been proposed to be the important anti-atherogenic particles within HDL which are involved in the removal of excess cholesterol from cells by a process which has been termed reverse cholesterol transport. LpA-I,A-II particles do not appear to be protective against the development of early CVD. We have initiated an study of LpA-I and LpA-I, A-II metabolism in normal controls and subjects with reduced and elevated levels of HDL cholesterol. Kinetic studies in normal subjects revealed that LpA-I was catabolized at a significantly faster rate than LpA-I,A-II. There was also a net conversion of LpA-I to LpA-I, A-II. Analysis of all of the kinetic studies performed with apoA-I and apoA-II revealed that the major determinant of plasma apoA-I levels is the rate of apoA-I catabolism whereas the major determinant of plasma apoA-II levels is the rate of apoA-II synthesis. These combined results support the concept that LpA-I and LpA-I,A-II may have different metabolic and functional roles in lipoprotein metabolism.

To further elucidate the mechanisms involved in LpA-I and LpA-I,A-II metabolism in both elevated and reduced levels of HDL, we have studied several kindreds with low (hypoalphalipoproteinemia) and high (hyperalphalipoproteinemia) levels of HDL. Of particular interest are patients with deficiencies of lecithin: cholesterol acyltransferase (LCAT), and cholesterol ester tranfer protein (CETP). LCAT is the plasma enzyme present on HDL (α LCAT) activity and apoB containing lipoproteins (β SCAT activity) which catalyzes the esterification of cholesterol to cholesterol esters. CETP exchanges cholesterol esters and triglycerides between HDL and VLDL/IDL/LDL.

B. Lecithin:cholesterol Acyltransferase

Patients with functional defects in LCAT present with remarkably different clinical features. In Classical LCAT deficiency the patients have a total deficiency of the LCAT enzymic activity, severe HDL deficiency, hemolytic anemia, cloudy corneas, and renal disease. In a second syndrome,

Fish Eye Disease (FED), the patients have a partial loss of LCAT activity, HDL deficiency, severe cloudy corneas, but no renal disease. We have identified two separate mutations, Tyr to Asn substitution at residue 156 and a stop codon at residue 83 which lead to Classical LCAT deficiency. In addition, we have elucidated three molecular defects in the LCAT gene which leads to FED. These include the substitutions of Thr-123 by Ile, Thr-347 by Met, and a triplet deletion resulting in the loss of Leu-300. In vitro expression and characterization of the variant LCAT enzymes have established the functional defects of these mutations and identified the molecular mechanisms for the reduced plasma activity of the LCAT enzymes. Kinetic studies were performed to determine the mechanism for the reduced levels of plasma HDL in both Classical LCAT deficiency and FED. In both types of LCAT deficiency, apoA-I and particularly apoA-II were catabolized substantially faster than in controls. Furthermore, LpA-I,A-II was catabolized faster than LpA-I, resulting in a selective decrease in levels of LpA-I,A-II. These results established that the reduced plasma HDL in both types of LCAT deficiency is due to increased catabolism. In addition, the preferential loss of the LpA-I,A-II particle but not the LpA-I particles may provide an explanation for the lack of an increased risk of premature CVD in LCAT deficiency despite the reduced levels of plasma HDL .

C. Cholesterol ester transfer protein deficiency.

A deficiency of CETP is an established genetic cause of hyperalphalipo-proteinemia. All of the known cases of CETP deficiency have been identified in Japan and have the same splice site mutation. The lipoprotein profile in subjects with CETP deficiency is characterized by markedly elevated levels of large HDL particles, reduced LDL, and triglyceride enriched HDL as well as LDL. Kinetic studies using endogenous labeling with amino acids labeled with stable isotopes were performed in order to determine the mechanism responsible for the elevated levels of HDL. LpA-I and LpA-I,A-II particles within HDL were isolated from three patients with CETP deficiency. The particles are larger, more lipid-enriched, and contained an increased number of apoE enriched HDL particles. LpA-I,A-II particles have a higher ratio of apoA-I to apoA-II. Kinetic studies revealed that the catabolic rates of HDL apolipoproteins A-I and A-II were substantially slower than in normal subjects. Thus the elevated levels of HDL are due to decreased catabolism. Both LpA-I and LpA-I,A-II from CETP deficient subjects have higher affinity but less binding capacity to Hep G2 cells when compared with normal HDL particles. These results are consistent with the decreased catabolism of the HDL particles. These findings are unique in that this is the first HDL dyslipoproteinemia in which there is delayed catabolism of HDL leading to hyperalphalipoproteinemia. These results are of particular importance since they suggest that the elevated levels of HDL may not protect against the development of premature CVD by reverse cholesterol transport but may be protective by a different mechanism.

Studies were also performed to determine the mechanism resulting in the low plasma levels of LDL and apoB. Kinetic analysis established that the LDL apoB was more rapidly catabolized in the subjects with CETP deficiency than in controls. In addition, LDL from a CETP deficient subject was not catabolized faster in normal subjects, suggesting that the LDL receptor is upregulated in this syndrome. Increased LDL receptor expression may be an important cause of the reduced plasma levels of LDL. Thus CETP deficiency significantly affects the metabolism of both HDL and LDL . Decreased catabolism of HDL and increased catabolism of LDL result in a lipoprotein profile that is less atherogenic. These findings extend our understanding of the effect of CETP in lipoprotein metabolism and are consistent with the concept that pharmacologic inhibition of CETP may be protective against premature atherosclerosis.

D. Lp(a)

Elevated plasma levels of Lp(a) are associated with an increased risk of premature CVD. Lp(a) is a lipoprotein similar to LDL that contains a unique apolipoprotein designated apo(a). The structure of apo(a) is similar to plasminogen. Apo(a) is polymorphic in plasma and the molecular

size as well as the plasma concentration in part is genetically determined. The other factors which influence Lp(a) levels are poorly understood. We have performed kinetic studies in individuals with the same apo(a) isoform but with widely different plasma levels of Lp(a). These studies established that differences in plasma concentrations were due to variations in the production rate of Lp(a) and not changes in catabolism. Thus research into other genetic factors independent of the apo(a) isoform may provide important insights into the mechanisms which determine plasma Lp(a) levels.

E. Familial Hypercholesterolemia

Patients homozygous for Familial Hypercholesterolemia (FH) have markedly elevated plasma cholesterol levels, arcus, tendon xanthomas, and an increased risk of premature CVD. The cause of the approximately 5 fold increase in plasma cholesterol and LDL cholesterol is due to a defect in the LDL receptor. Over the past 6 years, our Branch has prospectively evaluated the rate and progression of atherosclerosis by both invasive and non invasive techniques in 15 homozygote patients. Over the years these patients have been treated with diet, combination hypolipidemic drug therapy, portacaval shunting of the liver, plasma exchange, LDL apheresis, and/or liver transplantation. Each of the therapies has been partially successful in a selected patient. Studies will be continued to identify critical factors which mitigate the atherosclerosis present in these patients. In addition, our studies provide the framework for identifying patients which will benefit from gene therapy.

III Pathogenesis of Atherosclerosis

A. Pathophysiology of the early lesion of atherosclerosis

A major cell type that accumulates in atherosclerotic lesions is the plasma derived monocyte-derived macrophage. During the atherosclerotic process, macrophages in the vessel wall accumulate cholesterol as well as cholesterol esters and develop into foam cells. The foam cell is the characteristic cell of the early atherosclerotic lesion. Studies in the early stages of the atherosclerotic process have previously led to the identification of a pool of cholesterol-containing lipid particles that accumulate in the extra cellular spaces of atherosclerotic lesions. The particles are a mixture of unilamellar and multilamellar liposomes enriched with unesterified cholesterol (UC) and phospholipids. The UC-rich particles are unique in that they have a UC to phospholipid molar ratio >2:1. UC-rich particles appear after only 4 weeks of cholesterol feeding during early lesion development in rabbits. Studies with cholesterol loaded macrophages suggest that these UC-rich particles may be secreted from macrophages in the vessel wall. It is hypothesized that UC-rich particles are constituents of a basic metabolic pathway involving the reverse cholesterol transport of cholesterol from cells and tissues. HDL may play a central role in removing this cholesterol from the vessel wall and thus participate in reverse cholesterol transport.

B. Post-prandial atherosclerosis

Several studies have suggested selected individuals in the population appear to be susceptible to diet-induced atherosclerosis. A potential mechanism for the intestinal production of atherogenic particles is the increased production of enterocytically-derived apoB-100. We have previously established that 7-15% of the apoB synthesized by the human intestine is apoB-100, the rest being apoB-48. Since the plasma half-life of apoB-48 is less than 15 minutes and apoB-100 is more than 2 days, increased production of triglyceride-rich, longer half lived, atherogenic apoB-100 particles could represent an increased risk for early heart disease. Intestinal biopsies have been obtained from normal controls and patients with hypertriglyceridemia, delayed chylomicron clearance and a family history of premature CVD. The apoB isoform as well as the percentage of apoB mRNA editing were evaluated. One patient was identified who synthesized 3 fold more apoB-100 than

normals postprandially. These findings suggest that diet-induced atherosclerosis may be pathophysiologically related to genetic defects in apoB editing.

C. Experimental Atherosclerosis

A major breakthrough in the potential analysis of the factors which modulate the development of premature CVD has been achieved. A transgenic rabbit program has been initiated to systematically investigate the importance of genes modulating HDL metabolism and reverse cholesterol transport on experimental atherosclerosis. Genomic apoA-I was cloned and a series of constructs were prepared. Several constructs were injected into WHHL rabbits which have severe hypercholesterolemia and a defect in the LDL receptor. Transgenic rabbits which express apoA-I have been identified. The establishment of a transgenic rabbit program permits the direct testing of the reverse cholesterol transport hypothesis and the potential role that HDL plays in preventing premature atherosclerosis.

Annual Report of the
Molecular Hematology Branch
National Heart, Lung, and Blood Institute
October 1, 1991 to September 30, 1992

This is the final annual report of the Molecular Hematology Branch (MHB) under its present head. MHB has been composed of three sections: the Section on Molecular Genetics has primarily been involved in developing the basic knowledge and technology for carrying out gene therapy for human diseases; the Section on Molecular Cloning has primarily been concerned with understanding the nature of transcriptional control elements; and the Section on RNA and Protein Biosynthesis has primarily been concerned with understanding the mechanism and regulation of eukaryotic gene expression at both the transcriptional and translational levels. This past year the Section on RNA and Protein Biosynthesis has added a new responsibility, namely to attempt to learn the mechanism of site-specific integration of the Adeno-associated virus.

SECTIONS ON MOLECULAR GENETICS AND MOLECULAR CLONING

A number of human gene therapy clinical protocols are now underway. The first gene therapy protocol, for the treatment of adenosine deaminase (ADA), has been successful. The first patient to receive gene-corrected cells (in September, 1990) has been followed for nearly two years and has shown significant clinical improvement. A second patient is also doing well. Both patients have responded well to the regimen of infusions of autologous gene-corrected T-lymphocytes. A new protocol has been approved by the RAC in which autologous peripheral blood stem cells (the CD34-enriched cell population) would be gene-corrected and returned to the patient.

In addition to the ADA clinical protocol, several gene therapy protocols for the treatment of advanced cancer have been initiated. Seven patients have received autologous tumor infiltrating lymphocytes (TIL) containing a tumor necrosis factor (TNF) gene. This Phase 1 safety study is progressing well with no indications of any adverse side effects from the gene transfer procedure. Several patients have been started in tumor-vaccine trials where they receive autologous tumor cells into which has been inserted an expressing cytokine gene (either TNF or IL-2). These early trials are also going well.

The diseases chosen by the MHB as the initial candidates for human gene therapy are ADA deficiency, cancer (specifically,

malignant melanoma), AIDS, and hemophilia. ADA deficiency and cancer protocols are underway; an AIDS gene therapy clinical protocol is about to be submitted; and a new program designed to treat hemophilia has been initiated.

Retroviral techniques and recombinant DNA technology have been used to construct retroviral vectors containing a specific gene: for example, the human ADA gene, a cytokine gene (tumor necrosis factor, interleukin 2, alpha-interferon, etc.), the human soluble CD4 gene, and/or a selectable gene, for example the NeoR gene (which codes for a phosphotransferase enzyme that confers resistance to the drug G418, a neomycin analogue that can kill mammalian cells). An efficient procedure has been developed for transferring functional genes into mammalian tissue culture cells in vitro and into bone marrow cells and into T lymphocytes of mice and monkeys in vivo using these retroviral vectors as a retroviral-mediated gene transfer delivery system.

In past years we have demonstrated successful transduction in vitro of murine, sheep, monkey, and human hematopoietic progenitor cells as well as of fibroblasts, hepatocytes, endothelial cells and a number of other cell types from several different species. Although considerable effort is being made to carry out the human gene therapy clinical protocols, the major emphasis this past year has been in acquiring a greater understanding of the basic biology of these systems in order to be able to develop new and improved approaches for human gene therapy.

During the past year, the primary achievements of these Sections have been:

(1) The success of the ADA human gene therapy clinical trial.

(2) The initiation of several human gene therapy clinical protocols for the treatment of advanced cancer, not only at the NIH but also with collaborators in other institutions.

(3) Progress towards obtaining the preclinical data necessary to prepare clinical protocols for the treatment of other diseases, including AIDS (e.g., secretion of soluble IgG-CD4 from autologous gene-engineered cells), various genetic diseases (e.g., treatment of hemophilia with Factor 8 or 9), and cardiovascular diseases (e.g., by having gene-engineered vascular endothelial cells secrete t-PA in the area of an intravascular graft or newly inserted stent).

(4) Initial progress in developing an injectable, targetable retroviral vector as a delivery system for gene therapy. In addition, these Sections are collaborating with the Section on RNA and Protein Biosynthesis in the creation of a new unit to study the site-specific integration of Adeno-associated virus as a model for developing site-specific integration for gene therapy vectors.

SECTION ON RNA AND PROTEIN BIOSYNTHESIS

To understand the regulation of gene expression, promoter elements of the Adenovirus 2 major late transcription unit and the eukaryotic translation factor eIF-2 alpha gene are being used to characterize and purify individual factors required for accurate initiation. In addition, a new program has been initiated that is attempting to understand the mechanism whereby Adeno-associated virus is able to integrate into one fairly narrow region in human chromosome 19. The objective is to develop, in collaboration with the Section on Molecular Genetics, injectable gene therapy vectors that will integrate into a "safe" site in the human genome.

During the past year this section has generated the following major findings:

(1) A novel cis-acting regulatory sequence has been identified at the CAP site sequence (+1 to +22) in the Ad2 MLP. The transacting factor binding to this sequence has been designated the CAP-site binding factor (CBF). Mutational analysis of the CAP-site sequence shows that it is required for efficient transcription both in vitro and in vivo.

(2) Dnase I footprint analysis confirms that distinct polypeptides bind to the TATA box and CAP-site elements. These factors segregate during purification and binding to their cognate sequences can be individually competed by specific competitor oligonucleotides. These studies demonstrate, therefore, that the extended DNase I protection downstream of the TATA/CAP region is not the result of TFIID alone, but rather is contributed partly by the binding of a novel transcription factor binding to the CAP site region (+1 to +22).

(3) Translational activation, a major component of mitogenic stimulation of T-cells, occurs at the level of translational initiation. Contributing to this is a 50-fold increase in eIF-2 alpha mRNA. The mechanism of this large change is predominantly post-transcriptional, and appears to involve altered stability and/or processing of the primary transcript.

(4) The extent of eIF-4E phosphorylation also increases dramatically during mitogenic stimulation, and is probably a major cause of the exhibited translational activation.

(5) We have identified a DNA binding site in the eIF-2 alpha promoter region consisting of direct and inverted repeats and palindromic sequences by a variety of footprinting techniques. This binding site lies within a DNase I hypersensitive site in chromatin and overlies the multiple transcriptional start sites

of the eIF-2 alpha gene. The footprint consists of two nonoverlapping binding sites that bind the cognate protein noncooperatively and with differing affinities. By in vitro transcription using a dimer of the high affinity binding site upstream of the core adenovirus 2 major late promoter, this element confers a reproducible 2-3 fold increase in transcriptional activity.

(6) We have purified to near homogeneity a 66-68 kDa protein that binds to this element. This protein, designated α-PAL because of the palindrome sequences within its binding site, protects a region extending from -74 to -10 in in vitro Dnase I footprint experiments.

Annual Report of the Pathology Branch
Division of Intramural Research
National Heart, Lung, and Blood Institute
October 1, 1991, to September 30, 1992

The Pathology Branch focuses on structural alterations caused by various cardiovascular diseases. As in past years, the focus has been on structural changes produced in coronary, valvular, congenital, and cardiomyopathic heart diseases.

CORONARY ARTERY DISEASE

A number of studies have been done in the past in this Branch having to do with quantitative analysis of the amounts of coronary narrowing in various subsets of patients with fatal coronary artery disease, and also in groups of patients with various non-cardiac conditions such as systemic lupus erythematosus, Hurler's Syndrome, adult-onset diabetes mellitus, etc. It is well known that patients with abdominal aortic aneurysm often have symptomatic or asymptomatic atherosclerosis in other body organs. A major cause of death in patients with abdominal aortic aneurysm is coronary artery disease. Despite this common associated occurrence, however, the *amounts of coronary narrowing in patients with abdominal aortic aneurysm* has not been determined. We studied 27 patients with an abdominal aortic aneurysm which was 5.0 cm in widest transverse diameter or larger. During life, 12 (44%) of the 27 patients had myocardial ischemia and 10 of the 27 patients died from its consequences. Fifteen (56%) of the 27 patients had evidence of acute or healed myocardial infarcts. Of the 27 patients, 23 (85%) had narrowing >75% in cross-sectional area of 1 or more coronary arteries by atherosclerotic plaque. Of the 108 major coronary arteries in the 27 patients, 55 (51%) were narrowed >75% in cross-sectional area by plaque. The 4 major coronary arteries were divided into 5.0 mm segments. Of the resulting 1,475 segments, 18% were narrowed >75% in cross-sectional area. Thus, patients with abdominal aortic aneurysm, at least at necropsy, nearly always have diffuse and severe coronary atherosclerosis.

We also studied the *amounts of coronary narrowing in patients who had undergone amputation of 1 or both legs* because of severe peripheral arterial atherosclerosis. Of the 26 patients, 15 (58%) had symptoms of myocardial ischemia and 12 (42%) died from the its consequences. Of the 26 patients, 24 (92%) had narrowing 76-100% in cross-sectional area of 1 or more major coronary arteries by atherosclerotic plaque. The 4 major coronary arteries were divided into 5-mm segments and of the resulting 1,322 segments, 28% were narrowed >75% in cross-sectional area by plaque. Thus, patients with peripheral arterial atherosclerosis severe enough to warrant amputation nearly always have diffuse and severe coronary atherosclerosis at the time of necropsy.

In the last 3 years, several studies in this Branch have been concerned with composition of the atherosclerotic plaque in various subsets of coronary patients. One study in the above time period concerned *comparison of composition of atherosclerotic plaques in saphenous vein aortocoronary bypass conduits and in native coronary arteries in the same 19 men*, all of whom survived a coronary artery bypass operation for >1 year. The major finding in this study was that cellular fibrous tissue was the dominant component of the

plaques in the saphenous vein grafts whereas dense fibrous tissue was the dominant component of the plaques in the native coronary arteries in the same patients. Of the patients in whom the bypass operation was done from 14 to 26 months earlier, cellular fibrous tissue made up 80% of the plaques in the saphenous veins and only 13% of those in the native coronary arteries. As the survival time after the bypass operation increased, however, the composition of the plaques in the saphenous veins changed such that by 80 months, the amounts of cellular and dense fibrous tissue in both saphenous vein grafts in native coronary arteries were similar. Thus, by about 7 years after a coronary bypass operation, the composition of plaques in saphenous vein grafts is similar to that in the native coronary arteries in the same patients.

There is no morphologic information on the *composition of atherosclerotic plaques in men versus women.* Therefore, we did a study comparing composition of plaque in both saphenous vein aortocoronary bypass grafts and in native coronary arteries of 11 men and of 11 women in whom the time intervals between bypass operation and death were similar. A total of 979 five-mm segments of native coronary arteries and 842 five-mm segments of saphenous vein were examined. Analysis of the various plaque components revealed that the atherosclerotic plaques in women contained significantly more cellular fibrous tissue than those in men, both in the native coronary arteries and in the saphenous vein grafts. Dense fibrous tissue was significantly less in the plaques in the native coronary arteries in women compared to those in men and in the plaques of the saphenous vein grafts. Thus, plaque composition at least in the native coronary arteries and in aortocoronary bypass conduits is significantly different in men compared to women.

No information is available on the *composition of atherosclerotic plaques in the coronary arteries in patients with juvenile diabetes mellitus.* We studied the composition of atherosclerotic plaques in 331 five-mm segments of the 4 major coronary arteries in 8 patients with juvenile (mean age onset, 9 years; mean age of death, 29 years) diabetes mellitus. Analysis of the coronary segments disclosed that the plaques consisted primarily of dense and cellular fibrous tissue (53% and 38%) and that extracellular lipid occupied only 7% of the plaques and intracellular lipid only 1% of the plaques. Thus, 91% of the coronary plaques in these young diabetic patients consisted of fibrous tissue and nearly all of the remaining 9% consisted of lipid deposits. Compared to older patients with fatal coronary artery disease, the patients with juvenile diabetes had more dense fibrous tissue and extracellular lipid and less cellular fibrous tissue and calcific deposits.

Right ventricular myocardial infarction occurs in about 25% of patients with acute myocardial infarction involving the posterior wall of the left ventricle. We wondered *if thrombolytic therapy during acute myocardial infarction altered the frequency of right ventricular infarction.* We studied 51 patients who died after having received intravenous recombinant tissue plasminogen activator for left ventricular acute myocardial infarction. Right ventricular infarction occurred in none of the 29 patients with anterior wall left ventricular infarction but it occurred in 8 (36%) of 22 patients with posterior wall left ventricular infarction. The 8 patients with right ventricular infarction were compared to the 14 patients without right

ventricular infarction, all of whom had posterior wall left ventricular infarcts. Those with right ventricular infarcts had a longer mean interval from tissue plasminogen activator infusion to peak creatine phosphokinase level, a lower frequency of hemorrhagic necrosis, and a higher frequency of luminal thrombus in the infarct-related coronary artery. Each of these findings is associated with the absence of coronary reperfusion. Thus, it appears that successful reperfusion following acute myocardial infarction of the posterior wall of left ventricle is associated with a decreased frequency of concomitant right ventricular myocardial infarction.

VALVULAR HEART DISEASE

Patients with valvular aortic stenosis may have a unicuspid, a bicuspid, or a tricuspid aortic valve. We wondered if one could *predict the structure of the aortic valve in patients with aortic valve stenosis by knowing the patient's age, body weight, cholesterol level, and the amount of coronary narrowing by angiogram.* We studied 188 patients over 40 years of age at the time of aortic valve replacement, and all had coronary angiograms and determination of the peak systolic gradient across the stenotic valve preoperatively. Eighty patients (43%) had at least 1 coronary artery narrowed >50% in diameter. Forty-three patients (43%) underwent coronary artery bypass grafting at the time of aortic valve replacement. One hundred and twenty-three patients had a total cholesterol level >200 mg/dl and 48 patients had a body mass index >27 kg/m^2. A logistic regression model was developed which found 4 factors (age, total cholesterol level, significant coronary artery narrowing, and body mass index) to be predictive of aortic valve structure: 1) patients with coronary artery disease and a body mass index >27 kg/m^2 had a very low probability (5-20%) of having a congenitally malformed aortic valve; patients aged $\leq$65 years and a total cholesterol $\leq$200 mg/dl had a very high probability (85-91% of having a congenitally malformed valve). Thus, by knowing the patient's age, cholesterol level, degrees of coronary narrowing and body weight, it is possible to predict with reasonable accuracy the underlying valve structure in patients with stenotic aortic valves.

It is well known that patients, particularly those >40 years of age, with valvular aortic stenosis often have associated coronary narrowing. We reviewed 33 previously reported studies which described the *frequency of narrowing (>50% in diameter) of 1 or more coronary arteries by angiogram in patients with valvular aortic stenosis* and found that 37% of the reported patients (1302 of 3509) had significant narrowing by angiogram of 1 or more major coronary arteries.

Several years ago, a study from this Branch in patients with valvular aortic stenosis reported that total 12-lead QRS voltage is the best predictor of left ventricular hypertrophy in patients with valvular aortic stenosis. Since that initial study, we have also determined 12-lead QRS voltage in patients with amyloid heart disease, carcinoid heart disease, pure aortic regurgitation, mitral stenosis, idiopathic dilated cardiomyopathy, and hypertrophic cardiomyopathy. The latest study done in the above time period concerned *total 12-lead QRS voltage in patients pure mitral regurgitation.* We found that the highest sensitivity among the criteria for left ventricular

hypertrophy in this study group was the total 12-lead QRS voltage which was above normal in 71%. Other criteria for left ventricular hypertrophy were far less sensitive, including the Romhilt-Estes criteria (17% sensitive) and the Sokolow-Lyon index (46% sensitivity).

A study done a year ago in this Branch pointed out that not only is the mitral valve thicker than normal in patients with hypertrophic cardiomyopathy but also that its area is larger than normal and the length of the anterior mitral leaflet is larger than normal. A study done in this time period compared *the area, length and circumference of the mitral valve in hypertrophic cardiomyopathy to these mitral valve dimensions in patients with aortic valve stenosis, pure aortic regurgitation, and in a group of subjects without heart disease.* We found that the mitral leaflet area is commonly increased in size in patients with aortic valve disease with dilated left ventricles. In this circumstance, the mitral changes are secondary to the left ventricular cavity dilatation. In patients with hypertrophic cardiomyopathy, since their left ventricular cavities are of normal size or smaller than normal, the increased mitral dimensions could not be attributed to an increase in left ventricular cavity size. Thus, increased mitral leaflet area in hypertrophic cardiomyopathy may be due to a primary effect of this condition on the mitral valve.

CARDIOMYOPATHY

A number of studies have described disorganization of myocardial fibers in the ventricular septum in patients with hypertrophic cardiomyopathy and in some other varieties of congenital heart disease. We examined histologic sections of *ventricular septum in 47 patients with hypertrophic cardiomyopathy from the viewpoint of whether or not the muscle bundle in the midwall layer maintains its normal circular orientation surrounding the left ventricular cavity.* The circular orientation was lacking largely or completely due to marked fascicle disarray in 77% of the anterior and posterior junctional segments in the 47 patients. In 33% of the middle portion of the ventricular septum and 34% of the anterior and posterior portion of the left ventricular free wall, the midway layer showed disarray of muscle fibers of small fascicles. In contrast, the lateral left ventricular free wall was devoid of disarranged fibers. Destruction of the circular unit in the area of the junction of ventricular septum to right and left ventricular free wall in the patients with hypertrophic cardiomyopathy is considered a new morphologic aspect of this disease. Its functional significance, however, is unclear.

The left ventricular papillary muscles are believed to be the last portions of myocardium to be perfused with arterial blood. Patients with severe chronic anemia, therefore, might be expected to have some morphologic lesions in the papillary muscles as a consequence of inadequate perfusion. Therefore, we looked at the *hearts of 13 patients with sickle cell anemia to see if there were signs of inadequate myocardial oxygenation of the papillary muscles.* Nine of the 13 patients with sickle cell anemia had foci of fibrosis in the left ventricular papillary muscles and all of these patients were under 45 years of age.

CONGENITAL HEART DISEASE

During the last 10 or so years, a number of publications from this Branch have concerned congenital anomalies of the coronary arteries. We studied at necropsy 17 patients in whom the *left main coronary artery arose from either the right aortic sinus or the most proximal portion of the right coronary artery.* We found that the left main coronary artery coursed to the left side of the heart by 1 of 4 routes. In Group A, the anomalous left main coronary artery coursed anterior to the right ventricular outflow tract to reach the anterior sulcus where it divided into the left anterior descending and left circumflex coronary arteries. In 9 patients, the anomalously arising left main coronary artery coursed between (Group B) the ascending aorta and pulmonary trunk before reaching the anterior sulcus. In 2 patients, the anomalous artery coursed within the crista supraventricularis muscle behind the right ventricular outflow tract before reaching the anterior sulcus and in 4 patients, the anomalous left main coursed dorsal (Group D) to the ascending aorta before reaching the usual area of bifurcation into the left anterior descending and left circumflex coronary arteries. The only patients in whom the anomalous coursing artery was of clinical significance was the Group B group. In that group, the anomaly was usually fatal. In the other 3 groups, the anomaly was of no clinical significance.

Single ostium of a coronary artery in the aorta is rare. In a 33-year experience we found 10 patients at necropsy with solitary coronary ostium in the aorta. We also found previously published data in 35 necropsy-diagnosed cases and in 52 angiographically-diagnosed cases of solitary coronary ostium. Of the total 85 patients, 53 had absent or insignificant coronary luminal narrowing and 8 (15%) of them had clinical or morphologic evidence of myocardial ischemia. A classification of this complex anomaly was devised.

Normally, if the left circumflex coronary artery is hypoplastic, the right coronary artery is large, and when the left circumflex coronary artery is large, the right is hypoplastic. *Hypoplasia of both right and left circumflex coronary arteries* in the same patient is rare. We studied at necropsy 8 patients in whom bilateral hypoplasia was found, and, of them, 1 appeared to have died as a consequence of this anomaly. It appeared that 1 other patient had clinical evidence of myocardial ischemia as a direct consequence of bilateral hypoplasia. Bilateral hypoplasia of these 2 coronary arteries in the same patient has not been described previously.

SUMMARY

ULTRASTRUCTURE SECTION

Research studies in the Ultrastructure Section are centered on histologic, ultrastructural and immunohistochemical aspects of cardiovascular and pulmonary pathology. Studies are conducted on tissues from patients with clinically relevant disorders and from animals with experimentally induced lesions. This year, studies were completed on the pathology of the cardiac lesions associated with Chagas' disease; cardiovascular lesions resulting from toxic effects doxorubicin, amiodarone and interleukin-2; prosthetic heart valves including bovine pericardial bioprosthetic valves and cryopreserved aortic valve allografts; the effects of barotrauma on the structure of veins and venous grafts interposed in the arteriole circulation, and on the structure of pulmonary alveolar capillaries in patients with fibrotic lung disorders.

Cardiomyopathies

Chagas Disease

Chagasic cardiomyopathy, a consequence of infection with the parasite Trypanosoma cruzi, is the most prevalent type of chronic myocarditis in the world. It is characterized by progressive cardiac failure, cardiac dilatation, apical ventricular aneurysms and severe, recurrent arrhythmias. Morphologically, it is manifested by severe cardiac fibrosis, infiltration of the myocardium by lymphocytes and macrophages, myocyte-and degeneration, and thickening of the basement membranes of myocytes, endothelial cells and vascular smooth muscle cells. Many aspects of the pathogenesis of Chagasic cardiomyopathy remain to be elucidated, particularly with respect to the mechanisms causing myocardial damage and fibrosis. It appears that most of these alterations do not result from direct invasion of the myocytes by T. cruzi. Because of the importance of this cardiomyopathy, we have undertaken a series of studies of its pathology. Light and electron microscopic studies have been started on materials obtained by endomyocardial biopsy from patients with Chagasic cardiomyopathy and from mice and dogs with experimentally induced T. cruzi infection. The specific purpose of these studies is to define the nature of the interactions between lymphocytes and myocytes in the pathogenesis of the cardiac lesions. A study was completed of the structural alterations present in operatively resected ventricular aneurysms from three patients with Chagasic cardiomyopathy in whom refractory arrthmyias were demonstrated by electrophysiological mapping to originate from areas near the edges of the aneurysms. These studies revealed the presence of islands of severely altered myocardium (showing fibrosis, edema, mitochondrial damage and cellular hypertrophy) interspersed with normal myocardium in the aneurysmal walls, and it was concluded that such an arrangement of abnormally slow and normally conducting cells provides an anatomical basis for the occurrence of reentry types of arrhythmias.

Cardiac Toxicology

Doxorubicin Cardiotoxicity

Previous investigations from this unit have established that ICRF 187, a bis-dioxoketopiperazine, protects against the chronic cardiotoxicity produced in experimental animals by doxorubicin, a highly effective antineoplastic agent. The first human clinical trial of the cardioprotective effect of ICRF 187 was undertaken using patients with metastatic carcinoma of the breast. Our participation in this study, which was conducted at New York University by Dr. James Speyer and colleagues, consisted of evaluation, of endomyocardial biopsy specimens obtained from patients receiving doxorubicin, cyclophosphamide and 5-fluorouracil with or without ICRF 187. The severity of the cardiac morphologic changes produced by doxorubicin cardiotoxicity was evaluated in each specimen according to the

semiquantitative rating scale of Billingham. The results of this trial, which was completed this year showed clearly that ICRF 187 protects against doxorubicin-induced cardiotoxicity in terms of cardiac morphology and function. The use of ICRF 187 also allowed patients to receive much larger doses of doxorubicin without running the risk of the development of significant cardiotoxicity. No evidence was found to suggest that ICRF 187 interferes with the antitumor effects of doxorubicin.

Amiodarone Toxicity

To define the morphologic changes occurring in myocardium in patients receiving long-term treatment with amiodarone, an antiarrthymic agent, a survey was made of alterations in endomyocardial biopsy specimens from 50 patients with dilated cardiomyopathy who had been receiving long-term therapy with this drug. In two of these patients, the cardiac myocytes and endothelial cells contained cytoplasmic inclusions consisting of electron-dense concentric lamellae without a well defined periodic substructure. These inclusions were considered to represent intralysosomal accumulations of phospholipid and glycolipid material. Similar inclusions have been observed in other types of cells, including corneal epithelial cells and alveolar macrophages, in patients undergoing treatment with amiodarone. Inclusions showing this type of morphology have not been described previously in myocardium. Inhibition of lysosomal lipases by amiodarone is thought to be the cause of the accumulation of lamellar material observed in the present study. We suspect that the presence of lamellar deposits is not a simple function of the amount of amiodarone received by our 2 patients and that other, still undetermined factors may modulate the expression of this cellular abnormality, thus accounting for its infrequency among our patient population.

Toxicity of Interleukin-2

The administration of interleukin-2 (IL-2) for the treatment of certain neoplasms can result in a vascular leak syndrome characterized by hypotension, peripheral edema, pulmonary edema, hepatic damage and myocarditis. The pathogenesis of this syndrome is of considerable interest, because IL-2 induces the formation of lymphokine-activated killer cells (LAK cells) a type of lymphocytes capable of producing tissue damage. We have used mice and rats to develop animal models suitable for the study of the early stages of vascular leak syndrome induced by IL-2, and we have just completed a study of the interactions between lymphocytes and endothelial cells in this syndrome. These studies disclosed considerable damage to endothelial cells especially in venules and small veins of lung and liver. The endothelial cells in these areas showed cytoplasmic edema, vacuoles and myelin figures; in addition, there were frequent sites of transendothelial passage of lymphocytes which penetrated through their cytoplasm by means of "temporary migration pores" and accumulated in the perivascular spaces. The results of this study are consistent with other observations indicating that IL-2 activates both endothelial cells and lymphocytes, and that interaction between these two types of cells result in endothelial damage, with subsequent leakage of fluid into the extracellular space.

Prosthetic Heart Valves

Ionescu Shiley Bovine Pericardial Valves

Bioprosthetic cardiac valves constructed of glutaraldehyde-treated bovine parietal pericardium (Ionescu-Shiley valves) have been used extensively as replacement heart valves. However, structural failure has occurred in some of these valves after implantation in patients. The purpose of this investigation was to evaluate morphological changes found in Ionescu-Shiley valves removed because of structural failure four to eight years after implantation. Ionescu-Shiley valves explanted at reoperation from 17 patients in the Texas Heart Institute, Houston, Texas, were included in this study. A variety of morphologic changes were observed in these valves, including: primary tissue failure (with severe disruption of collagen) associated with the use of an alignment suture; thickening of valve leaflet; leaflet tissue

delamination; leaflet calcification, and dystrophic alterations of collagen. The most important of these findings was cuspal damage related to progressive enlargement of a hole produced by an alignment stitch placed near the margin of the commissure. This hole lead to eventual tearing of the pericardial leaflet. These findings indicate that valve design criteria directly influence the durability of pericardial valves; however, other factors unique to pericardial tissue also affect the durability and performance of these valves.

Studies on Cryopreservation of Allograft Valves

Cryopreserved human aortic valves are being used as replacement heart valves and have gained considerable popularity because of their long-term durability. This durability is considered to depend, at least in part on the viability of the cells in the graft. It is evident that this viability depends on the degree of postmortem autolysis which occurs during the time elapsed from death of the donor to the beginning of cryopreservation (herein defined as warm ischemic time). A study was undertaken to evaluate the extent to which reversible and irreversible injury occurs in cryopreserved human aortic valves as a function of warm ischemic time. Quantitative transmission electron microscopic observations were made to assess reversible and irreversible injury, according to well defined criteria, in 25 cryopreserved human aortic valves that had been harvested after various periods of warm ischemia. The results of the study showed that there was virtually no morphologic injury in valves with harvest-related warm ischemic times less than 2 hours and minimal irreversible cellular injury in valves exposed to 12 hours or less of warm ischemia. It was concluded that if cellular viability is critical to homograft durability, then harvest-related warm ischemia may need to be restricted to less than 12 hours.

Another study was made to define and quantify changes of reversible and irreversible cellular injury occurring in porcine aortic valves as a function of the duration of postmortem autolysis (herein referred to as warm ischemic time). Morphometric methods were used to analyze transmission electron micrographs of sections of valve leaflets harvested after periods of warm ischemia ranging from 40 minutes to 36 hours. The percentage of injured cells and the severity of both reversible and irreversible injury increased progressively with increasing duration of warm ischemia. After 24 hours irreversible injury was very severe and widespread. These findings emphasize the need for limiting warm ischemic times in order to maximize cell viability in cryopreserved heart valves to be used for valvular replacement.

Saphenous Vein Grafts

Effects of Barotrauma on The Structure of Veins and Venous Grafts in Monkeys

A study was made in macaques to determine whether veins subjected to barotrauma in situ undergo lipid uptake and morphologic changes to the same extent as veins grafted into the arterial circulation. The data obtained in this study showed that barotrauma alone does not cause veins that remain in the venous system to undergo the lipid uptake or morphologic changes that occur in veins used as arterial grafts.

Pulmonary Alveolar Capillaries in Fibrotic Lung Disorders

A study was made of the ultrastructure of pulmonary alveolar capillaries in open lung biopsy specimens from 29 patients with a variety of fibrotic lung disorders. In 20 of these patients the endothelial cells and capillaries differed structurally from those in normal capillaries in that they were of the fenestrated type. These cells were characterized by the presence of diaphragm-like cytoplasmic structures (fenestrate). Fenestrated and nonfenestrated endothelial cells are considered to be phenotypically different. Consideration is given to the possibility that the phenotypic change observed in the present study is a metaplastic process analogous to that which occurs in pulmonary alveolar epithelium in fibrotic lung disorders.

Annual Report of the Pulmonary Branch
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The research of the Pulmonary Branch centers on chronic hereditary and acquired disorders of the lung. All of these disorders are progressive, and usually fatal. Together, they affect more than 10 million individuals in the USA. In the past year, the Pulmonary Branch basic and clinical research program has been focused in three areas: I. Hereditary lung disease, including cystic fibrosis (CF) and α1-antitrypsin (α1AT) deficiency, the two most common lethal hereditary disorders in the USA; II. Gene therapy, including in vivo gene transfer strategies to treat lung disease; and III. Lung inflammation relevant to the pathogenesis and therapy of a diverse group of chronic lung diseases.

I. Hereditary lung disorders

CF is an autosomal recessive disorder resulting from mutations of the cystic fibrosis transmembrane regulator (CFTR) gene, a 27 exon gene occupying 250 kb of chromosome 7. All organs with exocrine glands are affected, but the major clinical manifestations are in the lung, with impacted mucus, chronic infection, inflammation, and airway and parenchymal lung derangements. The majority of studies in the past year regardng hereditary lung disease have focused on control of expression of the CFTR gene, and new therapeutic strategies (see also Section II "Gene Therapy") and Section III "Lung Inflammation" for CF and α1AT deficiency.

Consistent with the fact that CF is manifested on epithelial surfaces, active transcription of the CFTR gene and CFTR mRNA transcripts are detectable in a variety of epithelial cells, suggesting CFTR gene expression might be epithelial cell-specific. However, analysis of the CFTR gene promoter suggests it is a housekeeping gene, implying more widespread expression than only in epithelial cells. To evaluate the latter hypothesis, various human cells of non-epithelial origin, including lung fibroblasts, U-937 histiocytic lymphoma cells, K-562 erythroleukemia cells, HL-60 promyelocytic leukemia cells as well as freshly isolated blood lymphocytes, neutrophils, monocytes, and alveolar macrophages were examined for CFTR gene expression. Although Northern analysis failed to show CFTR mRNA transcripts in these cells, amplification of mRNA (after conversion to cDNA) by polymerase chain reaction combined with Southern analysis demonstrated the presence of CFTR mRNA transcripts at low levels in all cells evaluated except HL-60 cells. Comparative quantitative analysis showed fibroblasts contained 200-400 fold less CFTR mRNA transcripts than the T84 and HT-29 colon carcinoma epithelial cell lines, but had similar levels of CFTR transcripts to those of other epithelial cell lines. Nuclear transcription run-on analyses demonstrated very low level CFTR gene transcription in fibroblasts and U-937 cells, similar to that of other epithelial cells, but lower than the T84 and HT-29 colon carcinoma cell lines. Interestingly, while chromatin DNA of fibroblasts had no DNase I hypersensitivity sites in the 5′ flanking region of the CFTR gene, HT-29 chromatin DNA exhibited four DNase I accessible sites in the same region, suggesting that these sites may be related to more active transcription of the CFTR gene in the intestinal epithelial cells than in fibroblasts.

In CF, epithelial cells are unable to normally upregulate apical membrane Cl^- secretion in response to agents which increase cAMP, but do increase Cl^- secretion in response to increases in intracellular Ca^{++}. In the context that intracellular divalent cations regulate the expression of many genes, we hypothesized that mobilization of intracellular Ca^{++} and/or other divalent cations might modulate not only Ca^{++}-dependent Cl^- channels, but also CFTR gene expression. To evaluate this concept, HT-29 human colon carcinoma cells were cultured under various conditions designed to manipulate intracellular divalent cation concentration and CFTR gene expression was quantified at the level of transcription, mRNA accumulation, mRNA half-life and protein. Exposure to the divalent cation ionophores A23187 or ionomycin (agents which increase intracellular divalent cation concentration) caused a dose- and time-dependent reduction of CFTR mRNA levels, which could be blocked by use of Ca^{++}/Mg^{++}-free media. Ionophore-induced CFTR gene modulation was also observed in T84 human colon carcinoma cells and in freshly isolated normal human bronchial epithelial cells. Incubation of HT-29 cells with thapsigargin, an agent that releases Ca^{++} from intracellular stores, or in media containing increased extracellular concentrations of Ca^{++} or Mg^{++}, also caused a down-regulation of CFTR mRNA levels. Transcription run-on analysis showed that, parallel with the decrease in CFTR mRNA levels, A23187 reduced the rate of transcription of the CFTR gene while CFTR mRNA transcript half-life was unaffected. Consistent with the down-regulation of CFTR gene expression, CFTR protein levels also decreased after exposure to A23187. Thus, despite the independence of Ca^{++}-dependent Cl^- channels and cAMP-dependent CFTR-related Cl^- channels in epithelial cells, increases of intracellular divalent cation concentrations down-regulate the expression of the CFTR gene at transcriptional level with consequent decreases in CFTR mRNA and protein.

Epithelial cells utilize at least two types of apical Cl^- channel, the cAMP-activated CFTR channel and the Ca^{2+}/calmodulin dependent Cl^- channel. While phorbol ester (PMA) activates only CFTR-dependent Cl^- secretion and the Ca^{2+} ionophore A23187 only the Ca^{2+}/ calmodulin dependent Cl^- secretion, PMA and A23187 share the ability to down-regulate expression of the CFTR gene at the transcriptional level. Since both PMA and A23187 can activate protein kinases, we hypothesized that protein kinase pathways may be involved in the regulation of CFTR gene expression. Exposure of HT-29 human colon carcinoma cells to the protein kinase C activator SC9 down-regulated CFTR mRNA levels in a dose-dependent fashion, similar to that with PMA. The reduction in CFTR transcript levels by SC9 and PMA was blocked by H7, an inhibitor of protein kinases. In a similar fashion, the down-regulation of CFTR transcript levels by A23187 was blocked by H7 as well as staurosporine, another protein kinase inhibitor. Interestingly, both H7 and staurosporine themselves increased CFTR mRNA levels. Quantification of CFTR gene transcription rate showed a reduction by SC9 (similar to that with PMA and A23187) that was prevented by H7, and that H7 by itself increased CFTR transcription. Together, these observations suggest that protein kinase pathways, likely including protein kinase C, are involved in the regulation of CFTR gene expression, with activation or inhibition of protein kinase activity down-regulating or up-regulating CFTR gene expression, respectively.

Since CF is a recessive hereditary disorder, both parental CFTR genes must carry mutations for clinical disease to manifest i.e., only 50% of normal CFTR gene expression is required to maintain a normal phenotype. To help define the minimum amount of normal CFTR gene expression necessary to maintain normalcy, we have capitalized on our prior observation (EMBO J. 1991;10:1355-1363) that normal individuals can have up to 66% of bronchial CFTR mRNA transcripts that are missing exon 9, a region representing 21% of the sequence coding for the critical nucleotide (ATP)-binding fold 1 (NBF1) of the predicted CFTR protein. The study population included 78 individuals with no prior diagnosis of CF. Evaluation of bronchial epithelial cells (obtained by bronchoscopy) revealed that exon 9 was variably deleted in all individuals. Remarkably, there were four individuals, all $\geq$ 35 yr, in whom bronchial epithelial cells exhibited 73, 89, 90, and 92% CFTR transcripts with in-frame deletion of exon 9 respectively, despite normal sweat Cl^- and no clinical manifestation of CF. In the context that only 8% or less of bronchial CFTR transcripts need exon 9 to maintain normal airway function, these observations strongly suggest that either exon 9 is not necessary for CFTR structure and/or function or that only a very small fraction of bronchial epithelial cells need to express normal CFTR mRNA transcripts with exon 9 to perform the function of CFTR sufficient to maintain a normal phenotype in vivo.

Based on the knowledge that expression of the CFTR gene can be modulated at the transcriptional level, and that the CFTR gene promoter contains sequences homologous to elements in other promoters that respond to tumor necrosis factor-α (TNF), we evaluated the hypothesis that TNF might modulate CFTR gene expression in epithelial cells. Studies with HT-29 cells, a colon epithelium-derived tumor cell line known to express the CFTR gene, demonstrated that TNF downregulated CFTR mRNA transcript levels in a dose- and time-dependent fashion. Interestingly, nuclear run-on analyses demonstrated that TNF did not affect the rate of transcription of CFTR gene, but exposure of the cells to TNF did modify the stability of CFTR mRNA transcripts, resulting in a mRNA half-life that was reduced to 65% of the resting level. These observations suggest that CFTR gene expression can be modulated by TNF, at least in part, at the post-transcriptional level.

The respiratory manifestations CF are characterized by thick, infected secretions, neutrophil-dominated inflammation and progressive airway damage. Based on the knowledge that a variety of epithelial cells are capable of expressing the gene for interleukin-8 (IL-8), a potent inflammatory cytokine that attracts and activates neutrophils, we evaluated the hypothesis that mediators in respiratory epithelial lining fluid (ELF) of individuals with CF might induce IL-8 gene expression in epithelial cells, thus contributing to the airway inflammation. ELF was recovered by bronchoalveolar lavage from normals (n=7) and individuals with CF (n=8), incubated with the human bronchial epithelial cell line BET-1A, and IL-8 mRNA levels quantified. BET-1A cells at rest or incubated with normal ELF showed little IL-8 gene expression. In contrast, CF ELF induced BET-1A cells to express 1.8 kb IL-8 mRNA transcripts. The observations that CF ELF contained high levels of neutrophil elastase (NE) and a variety of serine protease inhibitors prevented CF ELF from inducing IL-8 gene expression in bronchial epithelial cells suggested that NE was the dominant mediator in

CF ELF inducing IL-8 gene expression. Consistent with this hypothesis, the addition of purified NE caused the BET-1A cells to express IL-8 transcripts in a dose- and time-dependent fashion and release of neutrophil chemotactic activity with characteristics of IL-8. Nuclear run-on analysis demonstrated NE upregulated IL-8 gene transcription in BET-1A cells, but did not affect the stability of the IL-8 mRNA transcripts, suggesting that upregulation of IL-8 gene expression by NE is modulated mainly at the transcriptional level. These observations were made in a transformed bronchial epithelial cell line but most likely represent the in vivo situation as evidenced by the absence of IL-8 in ELF from normal individuals and the presence of large quantities of IL-8 (8-108 μM) in CF ELF. This suggests a self-perpetuating inflammatory process on the CF bronchial surface where NE released by neutrophils induces the bronchial epithelium to secrete IL-8, which in turn recruits additional neutrophils to the bronchial epithelial surface.

Based on the knowledge that NE in CF ELF can induce human bronchial epithelial cells to express the gene for IL-8, we evaluated CF ELF for the presence of IL-8, and investigated the ability of aerosolized recombinant secretory leukoprotease inhibitor (rSLPI, a recombinant form of the naturally occuring bronchial NE inhibitor) to suppress NE, and hence IL-8, levels on the respiratory epithelial surface in CF. Western analysis demonstrated the IL-8 molecule in CF, but not in normal ELF. Quantitative enzyme linked immunoassay revealed 21.9 $\pm$ 4.8 nM IL-8 in CF ELF compared to undetectable levels in normals. Active NE was detectable in ELF of all individuals with CF. Aerosolization of rSLPI significantly raised SLPI levels in ELF ($p<0.01$) and significantly decreased active NE levels ($p<0.05$, all comparisons to pre-therapy). Human bronchial epithelial cells exposed to CF ELF recovered prior to rSLPI therapy expressed IL-8 mRNA transcripts, but ELF recovered from the same individual after rSLPI therapy produced markedly reduced bronchial epithelial cell IL-8 gene expression. Consistent with this in vitro observation, rSLPI aerosol therapy caused a marked reduction in CF ELF IL-8 levels ($p<0.05$), and comparison of CF ELF active NE levels and CF ELF IL-8 levels showed a remarkable association between the two parameters ($r=0.94$). Together, these data suggest that therapy of CF with a serine antiprotease such as rSLPI may not only suppress respiratory epithelial NE levels, but also break a cycle of inflammation on the CF epithelial surface, in which active NE stimulates bronchial epithelial cells to express the IL-8 gene and release IL-8, with subsequent attraction of more neutrophils, thereby increasing the burden of NE in the local milieu.

Airway secretions play a major role in the pathophysiology of CF. The secretions are thick, viscous, and difficult to expectorate, and they obstruct airways and contribute to reduced lung volumes and expiratory flow rates. The presence of high concentrations of DNA (up to 15 mg/ml) contributes importantly to their tenacious and viscous nature. The DNA is derived almost entirely from disintegrated inflammatory cells. Since it is an inherent property of DNA to form thick, viscous gels, purulent secretions in cystic fibrosis secretions have characteristics similar to those of a concentrated solution of DNA. Recombinant human deoxyribonuclease I (rhDNase) offers the possibility of specifically cleaving DNA in purulent

lung secretions, and may thus help patients with cystic fibrosis to remove secretions from their lungs. In vitro, rhDNase cleaves DNA in the sputum from patients with cystic fibrosis and reduces sputum viscosity. To evaluate the biologic activity and clinical efficacy of rhDNase in the short-term management of the lung disease of cystic fibrosis, we administered rhDNase by aerosol to patients with cystic fibrosis. The results of this preliminary study clearly indicated that rhDNase acts in vivo to cleave high molecular weight DNA in purulent lung secretions, and significantly improved lung function.

One process contributing to the airway derangement in CF is the chronic burden of oxidants released by inflammatory cells on the respiratory epithelial surface. With this background, we hypothesized that glutathione in CF respiratory ELF, might be oxidized and/or diminished in amount in CF ELF compared to normals. Recovery of ELF by bronchoalveolar lavage from young adults with CF (n=21) and normals (n=25) demonstrated marked neutrophil-dominated inflammation in CF ELF. As predicted, CF ELF was characterized by a deficiency of glutathione ($p<0.001$), but this was secondary to a reduction in reduced glutathione ($p<0.001$) as there were no differences in ELF levels of oxidized glutathione ($p>0.2$). Unexpectedly, there was also a marked deficiency of reduced glutathione in plasma ($p<0.02$), i.e., the glutathione "deficiency" observed in CF ELF is not limited to the site of the inflammation in CF, but is systemic. While this generalized deficiency of extracellular glutathione may be associated with chronic disease, it could be linked to the postulated function(s) of the CFTR gene product other than cAMP mediated Cl^- secretion.

Deletion of phenylalanine at amino-acid position 508 in CFTR (ΔF508 CFTR) is the most common mutation causing CF. It has been proposed that this mutation prevents glycoprotein maturation and its transport to its normal cellular location. We have expressed both CFTR and ΔF508 CFTR in Vero cells using recombinant vaccinia virus. Although far less ΔF508 CFTR reached the plasma membrane than normal CFTR, sufficient ΔF508 CFTR was expressed at the plasma membrane to permit functional analysis, ΔF508 CFTR expression induced a reduced activity of the cAMP-activated Cl^- channel, with conductance, anion selectivity and open-time kinetics similar to those of CFTR, but with much greater closed times, resulting in a large decrease of open probability. The ΔF508 mutation thus seems to have two major consequences, an abnormal translocation of the CFTR protein which limits membrane insertion, and an abnormal function in mediating Cl^- transport.

Transgenic mice bearing a human CFTR promoter-SV40 antigen fusion transgene were generated in order to localize in vivo the potential oncogenesis linked to the tissue-specific activity of the promoter for the CFTR gene. Surprisingly, the only site of tumors resulting from expression of the reporter onc gene was ependymal cells lining the brain ventricles. SV40 T antigen expression in these cells led to a consistent pathology in the first weeks of age: ependymoma and consequent hydrocephaly. Tumor-derived cell lines were established, characterized and shown to originate from SV40 T antigen-induced ependymoma. No pathological alterations were found in other organs, such as lungs and pancreas, in which CF is pathologically

manifest in humans. Such transgenic mice and derived cell lines may represent valid models for analyzing the role of SV40 T antigen in ependymoma formation and CFTR function in ependymal cells.

In order to examine the effect of nonsense mutations on CF gene expression, bronchial and nasal epithelial cells were obtained from a compound heterozygote patient for nonsense mutations R553X and W13I6X; a healthy normal individual; a patient homozygous for the common CF mutation (Δ508); and a CF patient who carries the R553X mutation and a missense mutation, S549N. When mRNA from bronchial cells of the normal individual, the Δ508 homozygote, and the S549N/R553X compound heterozygote was reverse transcribed and amplified by polymerase chain reaction using primers derived from the CF gene, DNA fragments of the predicted size were observed. However, the patient with nonsense mutations in each CF gene had no detectable CFTR messenger RNA, and therefore should have severely diminished and possibly absent CFTR protein. Furthermore, <2% of the CFTR transcripts in nasal epithelial cells from S549N/R553X compound heterozygote were derived from the gene with the nonsense mutations. We conclude that severe reduction in CFTR mRNA causes CF but can have different consequences in the lung and pancreas.

II. Gene therapy

Advances in the methods of molecular biology, and the understanding of the life-cycle of certain viruses, have opened the door to the feasibility of adapting gene transfer technology to modify the genetic program of the cells within the lung with the purposes of treating hereditary and acquired pulmonary disorders. Toward this end, the Pulmonary Branch has directed studies in the past year toward adapting recombinant adenoviruses to transfer genes in vivo to the lung and the liver, and in vitro to endothelial cells. Recombinant adenoviruses have been used to cointernalize DNA plasmids into cels in vitro, and plasmid-liposome combinations have been used to transfer genes into the lung in vivo.

Direct transfer of the normal CFTR gene to airway epithelium was evaluated using a replication- deficient recombinant adenovirus (Ad) vector containing normal human CFTR cDNA (Ad-CFTR). In vitro Ad-CFTR-infected CFPAC-1 CF epithelial cells expressed human CFTR mRNA and protein and demonstrated correction of defective cAMP-mediated Cl^- permeability. Two days after in vivo intratracheal introduction of Ad-CFTR in cotton rats, in situ analysis demonstrated human CFTR gene expression in lung epithelium. PCR amplification of reverse transcribed lung RNA demonstrated human CFTR transcripts derived from Ad-CFTR, and Northern analysis of lung RNA revealed human CFTR transcripts for up to 6 weeks. Human CFTR protein was detected in epithelial cells using anti-human CFTR antibody 11-14 days after infection. While the safety and effectiveness remain to be demonstrated, these observations suggest the feasibility of in vivo CFTR gene transfer as therapy for the pulmonary manifestations of CF.

To evaluate the diversity of airway epithelial cell targets for in vivo adenovirus-directed gene transfer, a replication deficient, recombinant adenovirus containing the E. coli lacZ [β-galactosidase (β-gal)] gene

(Ad.RSVβgal) was used to infect lungs of cotton rats. In contrast to uninfected animals, intratracheal Ad.RSVβgal administration resulted in β-gal activity in lung lysate and cytochemical staining in all cell types forming the airway epithelium. Strikingly, the distribution of the β-gal positive airway epithelial cells in Ad.RSVβgal infected animals was similar to the normal cell differential of the control animals. Thus, a replicant deficient, recombinant adenovirus can transfer an exogenous gene to all major categories of airway epithelial cells in vivo, cells, suggesting that adenovirus vectors may be an efficient strategy for in vivo gene transfer in airway disorders such as cystic fibrosis.

Replication deficient, recombinant Ad vectors do not require target cell replication for transfer and expression of exogenous genes and thus may be useful for in vivo gene therapy strategies in which hepatocytes are the target. In vitro, primary cultures of rat hepatocytes infected with Ad-αlAT, a recombinant Ad containing a human α1-antitrypsin (αlAT) cDNA, expressed human αlAT mRNA transcripts and synthesized and secreted glycosylated, functional human αlAT for 4 wk. In rats, in vivo intraportal administration of 10^{10} plaque forming units (pfu) of a recombinant Ad containing the E. coli lacZ gene, was followed by expression of the lacZ product (β-galactosidase) in 1% of hepatocytes 3d after infection. In vivo intraportal infusion of 10^{10} pfu of Ad-αlAT in rats resulted in average daily serum levels of human αlAT of 215-380 ng/ml for 4 wk. These observations suggest that in vivo intraportal administration of replication deficient, recombinant adenovirus vectors may be a useful strategy for in vivo gene therapy in disorders in which hepatocytes are the primary target for gene transfer.

To evaluate the feasibility of using a recombinant replication deficient adenovirus to transfer human genes to the human endothelium, human umbilical vein endothelial cells were infected in vitro with adenovirus vectors containing the lacZ gene coding for β-galactosidase, or a human α1-antitrypsin cDNA. Following in vitro infection with the lacZ adenovirus vector, cultured endothelial cells expressed β-galactosidase. In parallel studies with the αlAT adenovirus vector, infected cells expressed human αlAT transcripts as evidenced by in situ hybridization and northern analysis, and de novo synthesized and secreted glycosylated, functional αlAT within 6 hr of infection as shown by [^{35}S]methionine labeling and immunoprecipitation. Quantification of the culture supernatants demonstrated 0.3-0.6 μg human αlAT secreted/10^6 cells-24 hr, for at least 14 days after adenovirus vector infection. To demonstrate the feasibility of direct transfer of genes into endothelial cells in human blood vessels, lacZ or αlAT adenovirus vectors were placed in the lumen of intact human umbilical veins ex vivo. Histologic evaluation of the veins after 24 hr demonstrated transfer and expression of the lacZ gene specifically to the endothelium. αlAT adenovirus infection resulted in expression of αlAT transcripts in the endothelium, and de novo synthesis and secretion of αlAT. Quantification of αlAT in the vein perfusates showed average levels of 13 μg/ml after 24 hr. These observations strongly support the feasibility of in vivo human gene transfer to the endothelium mediated by replication deficient adenovirus vectors.

α1-antitrypsin deficiency, a hereditary cause of progressive emphysema, can

potentially be treated by transfer of a functional human αlAT gene to the respiratory epithelium. For such an approach to be successful, αlAT must be provided to both the interstitium and the epithelial surface i.e. the αlAT directed by the transferred gene must be secreted to both the apical and basolateral surfaces of the epithelial cells. To evaluate this concept, a recombinant, replication deficient adenoviral vector (Ad-αlAT) containing a human αlAT cDNA driven by an adenovirus major late promoter was used to infect Bet-1A human respiratory epithelial cells. The infected cells expressed Ad-αlAT directed mRNA transcripts and synthesized and secreted functional human αlAT as shown by [^{35}S]methionine labeling and immunoprecipitation of a 52 kDa glycosylated human αlAT molecule capable of interacting with neutrophil elastase, its natural substrate. Bet-1A cells grown on microporous polycarbonate membranes formed tight junctions (resistance >150 Ω x cm^2). After infection with Ad-αlAT, [^{35}S]methionine labeling and enzyme linked immunoassay demonstrated that αlAT was secreted into both the apical and basolateral compartments, with an average apical to basolateral ratio of 1.9 ± 0.2. Thus, human respiratory epithelial cells infected with a recombinant adenoviral vector containing a human αlAT cDNA secrete αlAT across both the apical and basolateral cell membranes, suggesting that the respiratory epithelium could serve as a target for in vivo gene therapy of αlAT deficiency.

Ongoing studies have demonstrated that the human Ad can augment transfer and expression of a gene within plasmid DNA unmodified by non-specific linkers or by linker-ligand complexes. Following the transfection of COS-7 cells with pRSVL, a luciferase expression plasmid vector directed by the Rous sarcoma virus-long terminal repeat promoter, luciferase activity in the target cells was 10^3 to 10^4-fold higher when the cells were also infected with Ad-CFTR, a replication deficient recombinant Ad containing human CFTR cDNA. The enhancement of luciferase gene expression in COS-7 cells was also observed with Ad-dl312 (a replication deficient Ela deletion mutant Ad with no exogenous gene) and wild type Ad5. The efficiency of cell transfection with pRSVL in the presence of an Ad was achieved in a dose-dependent fashion with progressively higher luciferase activity in cells infected by increasing amounts of Ad-CFTR, Ad-dl312 or Ad5. The augmentation by Ad-CFTR of the transfer and expression of the luciferase gene in cells was similar to that of another transfection reagent, cationic liposomes. Further, when Ad-CFTR and liposomes were used in combination, 4 to 100-fold more efficient expression of the luciferase gene was achieved than with Ad-CFTR or liposomes alone. Thus, exposure of target cells to replication deficient or competent human Ad will markedly augment transfer and expression of the genes within plasmid DNA in mammalian cells in vitro without modifying the plasmid with linkers or linker-ligand complexes, a strategy that should be useful for in vitro and in vivo gene transfer applications.

As another approach to gene therapy for the respiratory manifestations of CF, *in vivo* plasmid-mediated direct transfer of the normal CFTR gene to the airway epithelium was investigated in mice. To evaluate the feasibility of this strategy, pRSVL, a plasmid composed of a firefly luciferase gene driven by the Rous sarcoma virus long terminal repeat (RSV-LTR), along with cationic liposomes was instilled into the trachea of C57Bl/6NCR mice. With

administration of 200-400 μg plasmid DNA, luciferase expression could be detected in the mouse lung homogenates for at least 4 wk. With this background, a CFTR expression plasmid vector (pRSVCFTR) constructed by replacing the luciferase cDNA from pRSVL with the normal human CFTR cDNA was evaluated *in vivo* in mice. Intratracheal instillation of pRSVCFTR with cationic liposomes followed by analysis of mouse lung RNA by polymerase chain reaction amplification (after conversion of mRNA to cDNA) using a RSV-LTR specific sense primer and a human CFTR-specific antisense primer demonstrated human CFTR mRNA transcripts from one day to 4 wk after instillation. Further, *in vivo* evaluation of ß-galactosidase activity after intratracheal administration of an *E. coli lacZ* gene expression plasmid vector directed by the cytomegalovirus promoter (pCMVß) demonstrated that the airway epithelium was the major target of transfer and expression of the exogenous gene. These observations demonstrate successful plasmid-mediated gene transfer to the airway epithelium *in vivo*. This strategy may be feasible as a form of gene therapy to prevent the pulmonary manifestations of CF.

III. Lung inflammation

A large number of chronic lung disorders are characterized by chronic inflammation in the lung. The list of these disorders includes, CF, αlAT deficiency, cigarette smoking induced bronchitis and emphysema, interstitial lung disease such as idiopathic pulmonary fibrosis (IPF) and asbestosis, and granulomatous disorders such as sarcoidosis. In the past year, studies in the Pulmonary Branch have focused on inflammatory mediators, defenses against inflammation, and therapeutic strategies to prevent protease and oxidant damage.

The human bronchial epithelium is exquisitely sensitive to high concentrations of O_2, with tracheobronchitis usually developing 12 to 16 hr after exposure to 100% O_2. To evaluate the hypothesis that this vulnerability results from an inability of the bronchial epithelium to provide adequate antioxidant protection, we quantified antioxidant gene expression in bronchial epithelium of normal volunteers at baseline and after exposure to 100% O_2 <u>in vivo</u>. Following 14.8 ± 0.2 hr of 100% O_2, 24 of 33 individuals had evidence of tracheobronchitis. Bronchial epithelial cells recovered by brushing the airway showed no change in cell types or morphology, but the total number of cells recovered per brushing increased 36% after 100% O_2 ($p<0.03$), suggesting the cells were more loosely attached than normal. Quantification of baseline gene expression of CuZn superoxide dismutase (SOD), MnSOD, and catalase in bronchial epithelium demonstrated all were expressed at very low levels (CuZnSOD 4.1 ± 0.8 transcripts/cell, MnSOD 5.1 ± 0.9, catalase 1.3 ± 0.2) with control γ-actin expression relatively abundant (50 ± 6 transcripts/cell). Importantly, despite 100% O_2 exposure sufficient to cause tracheobronchitis in most individuals, there was no significant increase in any antioxidant mRNA transcripts/cell in the bronchial epithelium ($p>0.5$ compared to pre-O_2 levels). Together, the very low levels of expression of intracellular antioxidant enzymes and the inability to upregulate this expression with exposure to oxidant stress likely play a major role in the susceptibility of the human airway epithelium to hyperoxia.

Human neutrophil elastase (NE), a 29 kDa potent serine protease stored in azurophilic granules of mature neutrophils, is coded for by the NE gene, a single copy gene with 5 exons spanning a 6 kb segment of chromosome 11 at q14. With the knowledge that the NE gene expression is limited to early myeloid cell differentiation, mechanisms modulating expression of the NE gene were evaluated in the HL-60 promyelocytic leukemia cell line, a model of early bone marrow precursor cells. Consistent with the presence of NE mRNA transcripts in undifferentiated HL-60 cells, nuclear transcription run-on analyses demonstrated HL-60 cells actively transcribe the NE gene. However, the transcription rate of the NE gene was relatively low, only 40% of the myeloperoxidase gene, a gene expressed in parallel with NE. When induced toward the mononuclear phagocytic lineage with phorbol 12-myristate 13-acetate (PMA), HL-60 cells exhibited marked suppression of NE gene transcription, declining to 17% of the resting rate within 2 days. Induction toward mononuclear phagocytic lineage differentiation caused no change in NE mRNA transcript half-life ($T_{1/2}$), but mRNA levels decreased markedly over time, with levels undetectable 1.5 days after PMA stimulation. In contrast, when induced toward the myelocytic lineage with dimethyl sulfoxide, the rate of NE gene transcription increased 1.9-fold within 5 days. Interestingly, the mRNA transcript levels increased 2.5-fold by 5 days despite the fact that induction toward myelocytic lineage differentiation was accompanied by a marked reduction of NE mRNA transcript $T_{1/2}$. Together, these observations suggest that the NE gene expression during bone marrow differentiation is modulated mainly at the transcriptional level, with some posttranscriptional modulation contributing, particularly during myelocytic lineage differentiation.

Secretory leukoprotease inhibitor (SLPI), a 12 kDa serine antiprotease, normally serves to protect the upper airway epithelial surface from attack by neutrophil elastase (NE). In the context that a variety of inflammatory lung diseases are characterized by large neutrophil burdens with resultant high levels of NE in the lung, recombinant SLPI (rSLPI), a molecule identical to natural SLPI, may provide an effective means to augment the anti-NE protective screen of the lung. To determine whether intravenous rSLPI will augment respiratory tract and epithelial surface levels of SLPI and anti-NE capacity, rSLPI was administered intravenously to sheep and SLPI levels quantified in plasma, lung lymph (as a measure of lung interstitial levels), lung epithelial lining fluid (ELF) and urine. Following administration of 1 g rSLPI over 10 min, plasma levels of SLPI were 8 μM 30 min later, and decreased with a half life of 1.8 hr. Lymph SLPI levels paralleled the plasma levels; 4 hr after infusion the lymph/plasma ratio was 0.8. ELF SLPI levels paralleled the lymph levels; 4 hr after infusion the ELF/lymph ratio was 0.3. Western analysis demonstrated intact SLPI in lymph and ELF, and functional analysis showed increases in lymph and ELF anti-NE capacity that paralleled the levels of SLPI. As might be expected from a protein with a molecular mass of 12 kDa, urine excretion was high, with 20% of the SLPI excreted over 5 hr. However, if the rate of infusion was slowed, SLPI excretion decreased significantly, with a 3 hr infusion associated with 9% excretion and a 12 hr infusion <0.2% excretion. Importantly, slowing down the infusion rate was associated with only mild decrease in lung delivery, with ELF levels 2 hr following a 12 hr infusion 65% of the levels 2 hr after the 10 min infusion. Thus, intravenous rSLPI administration can mark-

edly augment the anti-NE defenses of the lung, and by slowing the rate of rSLPI administration, urinary excretion of SLPI can be markedly curtailed without consequent loss of delivery of the molecule to the target organ.

SLPI, serves as the major anti-neutrophil elastase (anti-NE) inhibitor on the epithelial surface of the upper airways. As a control for studies to evaluate the feasibility and safety of using aerosol administration of recombinant SLPI (rSLPI) to augment the anti-NE defenses of the lung, the status of antioxidants in respiratory epithelial lining fluid (ELF) was evaluated. Unexpectedly, we observed that aerosol administration of rSLPI caused an elevation in ELF glutathione, a major component of the epithelial antioxidant screen i.e., rSLPI may not only provide augmentation of anti-NE defenses, but antioxidant defenses as well. To evaluate this concept, rSLPI (100 mg) was aerosolized to sheep and SLPI, glutathione, anti-NE capacity, and anti-H_2O_2 capacity evaluated in respiratory ELF over a 30 hr period. As expected, aerosolization of rSLPI increased ELF SLPI levels and anti-NE capacity. Strikingly, post-aerosol levels of glutathione in ELF were also increased (5-fold 24 hr after aerosol), although rSLPI itself was undetectable in the glutathione assay, had no anti-oxidant activity in vitro, and no effect on plasma GSH levels. The rise in ELF glutathione following rSLPI aerosol was associated with an increase in ELF anti-H_2O_2 capacity i.e., the rSLPI augmented the antioxidant screen of ELF. As controls, aerosolization of saline or α1-antitrypsin did not increase ELF glutathione levels. Aerosolization of L-cystine also augmented ELF glutathione levels. Although this suggested the possibility that the rSLPI effect on ELF glutathione might relate to fragmentation of rSLPI, the aerosolization process did not alter the form of rSLPI. Further, while L-cystine augmented GSH secretion by bronchial epithelial cells in vitro, rSLPI did not. Intravenous administration of rSLPI also enhanced ELF glutathione levels, but not plasma glutathione levels. Thus, delivery of rSLPI to the respiratory epithelial surface augments both anti-NE and antioxidant local defenses, suggesting it may be particularly well suited for therapy in lung diseases characterized by excess of both serine proteases and oxidants on the respiratory epithelial surface.

Following the initial infection with HIV, there is evidence of immune dysfunction despite an apparent normal clinical state. In the context that the lung is a major site affected by opportunistic infection during the progression of this immune dysfunction, and that some components of the immune system are activated during early HIV infection, we hypothesized that there may be activation of alveolar macrophages (AM), a key component of the pulmonary host defense system, during the asymptomatic phase of HIV infection. Compared to normals, in HIV-infected individuals the class II MHC molecules DR, DQ, and DP were all expressed more frequently and in greater cell surface density on AM ($p<0.03$, all comparisons), and there was increased spontaneous release of superoxide anion ($O_2^{\cdot-}$) by AM ($p<0.002$). To gain insight into whether the activation of the AM was an inherent property of the cells or dependent on the in vivo milieu, AM were evaluated after 24 hr in culture for $O_2^{\cdot-}$ release. In contrast to the findings in fresh AM, after 24 hr in culture, $O_2^{\cdot-}$ release by HIV AM was not different from normals ($p>0.7$), suggesting that these AM had been activated in vivo. To assess whether IFN-γ could be mediating these effects, mRNA levels of the IP-

10 gene (a gene specifically induced by increased concentrations of IFN-γ) were quantified in AM. Strikingly, the IP-10 gene was expressed only in AM of HIV-seropositive individuals, suggesting the AM had been exposed to IFN-γ *in vivo*. Overall, these observations are consistent with the concept that the HIV-seropositive state is associated with activation of AM, in part due to local exposure to IFN-γ.

Concentrations of glutathione, a ubiquitous tripeptide with immune enhancing and antioxidant properties, are decreased in the blood and lung epithelial lining fluid (ELF) of human immunodeficiency virus (HIV)-seropositive individuals. Since the lung is the most common site of infection in individuals who progress to the acquired immunodeficiency syndrome, it is rational to consider whether it is possible to safely augment ELF glutathione levels in HIV-seropositive individuals, thus potentially improving local host defense. To evaluate the feasibility of this concept, purified reduced glutathione (GSH) was delivered by aerosol to HIV-seropositive individuals (n=14), and the glutathione levels in lung ELF were compared before and up to 3 hr after aerosol administration. Pre-therapy, ELF total glutathione concentrations were about 60% that of controls. Following 6 twice daily doses of 600 mg GSH, ELF total glutathione levels increased and remained in the normal range for at least 3 hr after therapy. Strikingly, even though >95% of the glutathione in the aerosol was in its reduced form, the percentage of oxidized glutathione in ELF increased from 5% before therapy to about 40% 3 hr after therapy, likely reflecting the utilization of glutathione as an antioxidant *in vivo*. No adverse effects were observed. Thus, it is feasible and safe to use aerosolized GSH to augment the deficient glutathione levels in the lower respiratory tract of HIV-seropositive individuals, suggesting it is rational to evaluate the efficacy of this tripeptide in improving host defense in HIV-seropositive individuals.

Various human lung diseases are characterized by an increased oxidant burden on the respiratory epithelial surface. As a step toward developing a therapy to augment the antioxidant defenses of respiratory ELF of the human lung, we have evaluated the feasibility of aerosolizing a human protein antioxidant to the respiratory epithelial surface of an experimental animal sufficiently large to permit repetitive sampling of ELF. To accomplish this, recombinant human Cu^{++}/Zn^{++} SOD (rSOD) was aerosolized to sheep and the levels of human SOD and anti-superoxide anion ($O_2^{\cdot-}$) capacity quantified in ELF over time. *In vitro* aerosolization did not alter the specific activity of rSOD ($p>0.5$). *In vivo* aerosolization of rSOD (100 mg) to sheep (n=7) resulted in peak amounts of human Cu^{++}/n^{++} SOD in ELF of 3.1 ± 0.6 μM, with a parallel increase in the anti-$O_2^{\cdot-}$ capacity of ELF. For the duration of the study (5 hr), SOD and anti-$O_2^{\cdot-}$ levels in ELF remained elevated, with a value 50% of peak at 5 hr. Aerosolization of phosphate buffered saline (n=5) had no effect on SOD or anti-$O_2^{\cdot-}$ levels in ELF. In animals receiving rSOD, there was no change in the specific activity of SOD recovered in ELF compared to the starting material ($p>0.4$). We conclude that rSOD can be delivered by aerosol to the ELF of a large animal with preservation of specific activity, and that a substantial increase in both amount of SOD and anti-$O_2^{\cdot-}$ capacity can be achieved for a period of time applicable to human therapy, supporting the rationale for evaluation of rSOD aerosol as an antioxidant in human pulmonary disease.

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Mouse Homeobox Genes. Homeobox and Pou-domain proteins bind to DNA and regulate gene expression. Hox-1.11 cDNA and genomic DNA were cloned and 5,856 bp were sequenced. The Hox-1.11 gene contains 2 exons separated by a small intron. The Hox-1.11 protein, 372 amino acid residues long, contains a conserved pentapeptide, a homeodomain, and an acidic region. The amino acid sequence of the Hox-1.11 homeodomain is identical to that of Hox-2.8, which suggests that both homeobox proteins may bind to the same or similar nucleotide sequences in DNA. In collaboration with C. Kozak the Hox 1.11 gene was mapped to mouse chromosome 6, which contains the Hox-1 cluster of homeobox genes. One species of Hox-1.11 poly A^+ RNA (1.7 kb) was found that is most abundant in 12-day-old embryos and progressively decreases thereafter. The most anterior expression of Hox-1.11 poly A^+ RNA in 12 to 14-day-old mouse embryos is the hind brain up to, but not including, the pons. Hox-1.11 poly A^+ RNA also is expressed in the spinal cord, the VIIth and VIIIth cranial ganglia, spinal ganglia, larynx, lungs, vertebrae, sternum, and intestine.

In collaboration with Y. Kim genomic DNA and cDNA for a novel mouse homeobox gene, mNK-1, were cloned and approximately 5.2 kb was sequenced. mNK-1 is the mouse equivalent of the Drosophila homeobox gene, NK-1, which we discovered previously. Fifty seven of the 60 amino acid residues of mNK-1 homeodomain are identical to the amino acid residues of the Drosophila NK-1 homeodomain (95% homology), which suggests that mouse and Drosophila homeodomain proteins bind to similar nucleotide sequences in DNA. Both mouse and Drosophila NK-1 proteins contain an acidic domain. mNK-1 poly A^+ RNA was detected in 12 to 18-day-old mouse embryos.

Hox 4.1 cDNA and genomic DNA were cloned and sequenced. The deduced amino acid sequence of Hox 4.1 protein consists of 417 amino acid residues. Hox 4.1 protein contains a conserved pentapeptide and a homeodomain that is similar to that of Hox 2.7 (97% homology). Comparison of Hox 4.1 and Hox 2.7 proteins revealed 59% homology, which suggests that these genes evolved from a common ancestor. Another homeobox gene, Hox 4.9, also was cloned and partially sequenced.

Pou-Domain Genes. The Pou-domain is a conserved amino acid sequence approximately 150 amino acid residues long that contains a Pou-specific domain and a homeodomain; both domains are required for high-affinity binding to DNA. Genomic DNA was cloned for four Pou-domain genes that are expressed in embryonic and adult mouse brain; Brain-1 (10.3 kb sequenced), Brain-2 (4.6 kb sequenced; Brain-2 cDNA also was cloned and sequenced), Brain-4, a novel Pou-domain gene, (3.2 kb sequenced), and Scip (9.9 kb sequenced). The four proteins have similar Pou-domain amino acid sequences; sequences in other regions of the proteins also are similar, but

to a lesser extent. Although the proteins contain between 361 to 495 amino acid residue, no introns were detected in the coding regions of the four Pou-domain genes. These results suggest that the 4 Pou-domain genes arose by duplication of an ancestral Pou-domain gene, which originated by reverse transcription of a molecule of Pou-domain mRNA followed by insertion of the cDNA into germ cell DNA.

Drosophila Homeobox Genes. Previously, we cloned a novel Drosophila homeobox gene, NK-2, which initially is expressed by virtually all cells in the ventral neurogenic anlage early in embryonic development when the ventral neurogenic anlage first appears. The results suggest that expression of the NK-2 gene results in cell commitment to the neuroblast pathway of differentiation, which is required for the development of a large portion of the CNS. During further development, only 20% of the cells continue to differentiate as neuroblasts; whereas 80% switch to the epidermoblast pathway of development and give rise to the ventrolateral epidermis of the embryo. Concomittantly, NK-2 mRNA levels decrease in some, but not all, neuroblasts resulting in a pattern consisting of clusters of neuroblasts with high levels of NK-2 mRNA surrounded by cells destined to become epidermoblasts with lower levels of NK-2 mRNA. During further development, expression of the NK-2 gene is extinguished in epidermoblasts and isolated precursors of neuroblasts with high levels of NK-2 mRNA can be seen surrounded by epidermoblasts with little or no NK-2 mRNA. It is likely that the positions in the embryo of the clusters of neuroblast precursors with high NK-2 mRNA are selected by a set of transactivating proteins. Then 1 neuroblast precursor per cluster is selected by lateral inhibition mediated by cell·cell interactions. Ten genes have been identified by others that are required for the neuroblast selection process. By defining the mechanisms that regulate NK-2 gene expression in future studies, we hope to understand how the number of neuroblasts and their relative positions are selected in a large part of the CNS.

A peptide that contains the NK-2 homeodomain was synthesized in E. coli and purified to apparent homogeneity. Most of the peptide was given to D. Tsao and J. Ferretti to determine the conformation of the NK-2 homeodomain in solution by nuclear magnetic resonance spectroscopy. The NK-2 homeodomain peptide also was covalently coupled to Sepharose and synthetic double-stranded oligodeoxynucleotides that contain random sequences in the middle were passed through the column under conditions that promote NK-2 homeodomain·DNA binding. Oligodeoxynucleotides with sequences that bind to the NK-2 homeodomain were purified by repetitive affinity-column chromatography, cloned, and sequenced, and a consensus binding sequence for the NK-2 homeodomain was defined. Putative NK-2 homeodomain binding sites were identified in the 5'-upstream region of the NK-2 gene, which suggests that NK-2 protein may regulate the expression of the NK-2 gene. Other putative genes regulated by NK-2 protein were purified by passing Drosophila genomic DNA fragments through the NK-2 homeodomain affinity-column. DNA retained by the column was eluted, cloned, and 42 clones of genomic DNA were sequenced partially. Multiple

binding sites for the NK-2 homeodomain were found in all sequences examined. Further work is needed to determine whether the cloned DNA fragments correspond to genes that are regulated by the NK-2 homeodomain protein *in vivo*.

P-Element Transposition. Transgenic lines of *Drosophila* were generated previously by transposition of a P-element that contains the β-galactosidase gene from one site in the genome to another. Some P-element insertions into genes expressed only in the nervous system were identified as homozygous lethal mutations. P-element insertions into some genes were found that result in gross morphological defects in the developing nervous system. The locations of about 60 genes with P-element insertions were mapped. P-elements with a few kb of adjacent *Drosophila* genomic DNA were cloned from 20 of the most interesting transgenic fly lines and the cloned *Drosophila* DNA genomic DNA fragments were used as probes to screen a *Drosophila* genomic DNA library for larger DNA fragments. Thus far, large genomic DNA clones were obtained that correspond to P-element insertion sites for 5 transgenic fly lines. Additional studies are in progress to characterize the genes that have been cloned that are expressed specifically in the nervous system.

Regulation of a Gene for a Voltage-Sensitive Calcium Channel α-1 Subunit. The efficiency of transynaptic communication between NG108-15 cells and cultured striated muscle cells previously was shown to be regulated by intracellular levels of cAMP or by retinoic acid, which in turn regulate the level of mRNA for a voltage-sensitive calcium channel α-1 subunit required for stimulus-secretion coupling. The 5'-upstream region of the calcium channel gene was cloned and sequenced and a novel trinucleotide repeat sequence was found that is a powerful activator of an enhancerless chloramphenicol acetyltransferase reporter gene. Synthetic oligodeoxynucleotides inserted upstream of the reporter gene in + or - orientations or inserted downstream of the reporter gene activate reporter gene expression. In collaboration with T. Kamp and E. Marban, a nuclear protein was found that binds to the novel enhancer sequence.

Enhancer Selection: Previously we devised a selection method for mouse genomic DNA clones that contain enhancer sequences based on the demonstration that the synthesis of polyoma virus DNA in mouse cells requires viral enhancer sequencers that also are required for the synthesis of mRNA from polyoma genes. An *E. coli*-mammalian cell shuttle vector was constructed that contains a library of mouse genomic DNA fragments ligated to the vector to replace the deleted polyoma virus enhancer region. In addition, the β-lactamase gene and *E. coli* origin of replication from pBR322 were inserted in the polyoma coat protein gene. Thus, only plasmids with mouse genomic DNA inserts that contain enhancers that activate plasmid DNA synthesis replicate in mouse cells and are selectively amplified. In collaboration with W. Odenwald, the enhancer selection method was used to clone genes that may be regulated by mouse homeobox protein, Hox-1.3. Mouse fibroblasts were cotransfected with a mouse genomic DNA library inserted in the polyoma shuttle vector and Hox-1.3 cDNA under the control of

constitutive enhancer sequences so that Hox 1.3 protein is synthesized. Two of the DNA clones that were obtained after several rounds of selection were sequenced. One clone contained 22 and the other 44 putative Hox-1.3 binding sites. Insertion of the cloned DNA upstream of a chloramphenicol acetyltransferase reporter gene was found to inhibit the expression of the reporter gene in cells that were cotransfected with the reporter gene construct and Hox-1.3 cDNA. Inhibition of reporter gene expression required the homeobox region of Hox-1.3 cDNA, which suggests that the inhibition of gene expression is dependent on Hox 1.3 homeodomain binding to DNA.

Previously, 3 novel mouse enhancer DNA sequences were found by the enhancer selection method. A cDNA expression library was screened for DNA binding proteins that bind with specificity to a 32P-labeled oligodeoxynucleotide enhancer sequence. cDNA clones were obtained that encode proteins that bind to each enhancer sequence.

Induction of the Proenkephalin Gene in Rat Liver. In cholestatic liver disease, plasma enkephalin levels are elevated, and the cholestasis-associated itching is ameliorated by opiate antagonists, which suggests that endogenous opioid peptide biosynthesis, degradation, or release may be altered in cholestasis. To test this hypothesis, levels of mRNA were measured for the 3 opioid peptide precursors in the adult rat and human liver, which normally does not synthesize opioid peptides. Experimental cholestasis in rat elicted by bile duct resection was found to strongly induce proenkephalin mRNA, but not prodynorphin or proopiomelanocortin mRNAs, in liver. In situ hybridization revealed that proliferating bile ductular cells, synthesize proenkephalin mRNA, rather than hepatocytes.

Proenkephalin Gene Transactivation by the HTLV-1 Tax 1 Protein. The transactivator tax 1 protein of the human T-cell leukemia virus I (HTLV-I) was found to transactivate the proenkephalin gene in a glial cell line. Sequences between bases -437 and -190 relative to the transcription start site were necessary for maximal transactivation. This finding may be relevant to diseases resulting from HTLV-I infection, such as T-cell leukemia and tropical spastic paraparesis, in which proenkephalin producing cells are infected.

Proneuropeptide Y Gene Regulation. Studies on the regulation of the gene coding for the precursor of neuropeptide Y, a peptide neurotransmitter in both central and peripheral nervous systems, revealed a cell-specific cAMP/phorbol ester inducible element in the 5'-flanking sequence of the rat neuropeptide Y gene between bases -2300 and -1800. However, sequences outside the region of the promoter tested (-2300 to +17) apparently are responsible for neuron-specific expression of this gene.

Distribution of Tropomodulin in Myofibrils. The distribution of tropomodulin in isolated myofibrils from rat and chicken skeletal muscle was determined using immunocytochemistry at the light- and electron-microscopic level. Tropomodulin was found to be

specifically associated with the pointed ends (in the A band) of the actin thin filaments in rat myofibrils, with both the pointed ends and the barbed ends (at the Z disk) of the thin filaments in chicken myofibrils. These results show that tropomodulin is associated with the ends of actin thin filaments at a stoichiometry of 1 to 2 tropomodulin molecules per filament. The results suggest that tropomodulin has a role in regulating thin filament length and/or is involved in crosslinking the thin filament ends.

Development of the Excitation-Contraction Coupling System. Developing triads containing ryanodine receptors (calcium release channels) and dihydropyridine receptors were found by immunocytochemistry and electron microscopy to be concentrated around nuclei in 3 day old myotubes. Rhythmic spontaneous contractions and corresponding calcium transients in 7 day old myotubes were found to be correlated with the appearance of triads throughout the myotubes.

Postsynaptic Aggregation of Acetylcholine Receptors. Monoclonal and polyclonal antibodies were obtained that are directed against a protein purified 4,000-fold from fetal pig brain that induces the formation of acetylcholine receptor aggregates on skeletal muscle cells in culture. The antibodies are being used to further purify and characterize the receptor aggregation protein and to determine the histological location of the protein and its relationship to a previously described protein, agrin.

Site-Specific Mutagenesis of Putative ATP Binding Sites in E. coli Adenylyl cyclase. Replacement of lysine 90 of E. coli adenylyl cyclase by either methionine or glutamine had a minor effect on the Vmax; however, the Km for the glutamine mutant was reduced three-fold. In contrast, the replacement of lysine 196 by methionine reduced enzyme activity to 0; whereas, replacement of lysine 196 by glutamine reduced enzyme activity 10-fold and reduced the Km three-fold.

Stimulation of Adenylyl Cyclase Activity by Nucleotides. When cells of E. coli are permeabilized with toluene, it is possible to demonstrate an adenylyl cyclase activity that is stimulated as much as six-fold by GTP. Studies of the kinetics of the adenylyl cyclase reaction in these preparations showed that velocity vs. substrate (ATP) concentration plots are sigmoid in the absence of GTP and hyperbolic in the presence of GTP. These data suggest an allosteric regulatory site that can be occupied by either ATP or GTP. Other naturally occurring ribonucleoside triphosphates (namely UTP and CTP) also promote the allosteric stimulation of adenylyl cyclase activity. Studies using mixtures of the ribonucleoside triphosphates indicated that there is only a single regulatory site for all the ligands. Nucleoside diphosphates are ineffective as regulators - a nucleotide triphosphate is required. Previous studies have suggested that proteins of the phosphoenolpyruvate:sugar phosphotransferase system (PTS) may interact with adenylyl cyclase to form a regulatory complex. Permeabilized cells prepared from a strain deleted for the genes for the general PTS proteins HPr, Enzyme I, and Enzyme III^{glc} show

no allosteric regulatory properties for adenylyl cyclase activity that is stimulated by GTP. The conclusion from these studies is that adenylyl cyclase interacts with PTS proteins to form an allosteric enzyme that can be regulated by GTP.

Involvement of Enzyme I of the Phosphoenolpyruvate:Sugar Phosphotransferase System as an Activator of Adenylyl Cyclase in E. coli. Permeable cells of E. coli allow the phosphocarrier HPr (M_r = 8,000) to pass in and out of the cells while Enzyme I (M_r = 70,000) is impermeable. We reconstituted PEP-dependent PTS activity in a PTS deletion strain that produces intracellular Enzyme III^{glc}, but not Enzyme I and HPr. In contrast, permeable cells of a strain that produced intracellular Enzyme I, HPr and Enzyme III^{glc} elicited PEP and Pi stimulated activity. These experiments add further weight to the argument that Enzyme I of the PTS is important not only as a phosphate donor to Enzyme III^{glc} but also as a regulatory protein in the adenylyl cyclase complex.

SUMMARY REPORT OF THE LABORATORY CHIEF

Laboratory of Biochemistry
National Heart, Lung, and Blood Institute
October 1, 1991 to September 30, 1992

Section on Enzymes

Protein Folding and Subunit Assembly

Genetically engineered protein products are often biologically inactive because of defects in the secondary, tertiary and quaternary structure. Protein factors, called chaperonins, aid in the correct folding of monomeric subunits and in their subsequent assembly to form functional multisubunit aggregates. In studies with *Escherichia coli* glutamine synthetase, it was shown that the chaperonin, groEL, increases the amount of the active dodecamer that can be obtained from denatured subunits. Interaction of groEL with glutamine synthetase monomeric subunit prevents incorrect folding of the subunit and thus increases the steady-state level of dimerization- competent monomers. Dissociation of the groEL-monomer complex is mediated by the binding of ATP and its conversion to ADP. The ATP-dependent dissociation of the groEL-monomer complex is facilitated by a second chaperonin, groES. Thus, working together, groEL and groES facilitate dimerization of correctly folded monomers which is the rate-limiting step in subunit aggregation.

Oxygen Toxicity

Some pulmonary deficiency disorders are likely due to damage caused by exposure of lungs to ozone in the atmosphere. As part of an on-going program to elucidate the chemistry of ozone toxicity, a sample of total cellular protein in which only histidine residues were exclusively labeled with radioactive carbon was obtained by growing *E. coli* on a nitrogen-rich medium containing radioactive histidine. Upon exposure to ozone, some amino acid residues in the total protein fraction were converted to carbonyl derivatives, and 90% of the histidine residues were destroyed; 50% of the missing histidine was converted to radioactive aspartic acid.

Reactions of Proteins with Lipid Oxidation Products

(a) ***Reaction with low density lipoprotein (LDL) with 4-hydroxynonenal (HNE).*** The oxidative modification of low density lipoprotein (LDL) by endothelial cells, smooth muscle cells, macrophages, and by metal ion catalyzed reactions is likely involved in the pathogenesis of atherosclerosis. 4-Hydroxynonenal (HNE), an important cytotoxic product of polyunsaturated fatty acid oxidation, was found to react with histidine, and lysine residues of the protein moiety of LDL. A role of HNE in the oxidative modification of LDL was confirmed by the demonstration that HNE adducts of histidine and lysine residues in LDL are formed during the oxidation of LDL by O_2 and cupric ions. Moreover, polyclonal antibodies directed against HNE-histidine adducts of histidine-containing polypeptides were shown to react with oxidized LDL.

(b) ***Reaction with glyceraldehyde-3-phosphate dehydrogenase (GAPDH).*** Reaction of HNE with GAPDH leads: to loss of catalytic activity; to the modification of several histidine, lysine and cysteine residues; to the formation of both intra- and inter-protein cross-linkages; and to the generation of carbonyl groups. It is concluded that the reactions with HNE involve a Michael type mechanism in which the imidazole nitrogen, amino group and sulfhydryl group of histidine, lysine and cysteine residues, respectively, add to the α,β-double band of HNE to form protein bound aldehydes, which can undergo secondary Schiff base reactions with other lysine residues to form cross-linked derivatives.

(c) ***Reaction of HNE with glucose-6-phosphate dehydrogenase (GPDH).*** Reaction of the GPDH from *Leuconostoc mesenteroides* with HNE leads to inactivation of the enzyme, and to the formation of a protein carbonyl group. The loss of activity is due to a Michael type reaction of HNE with the ϵ-amino group of a lysine residue, that is involved in the binding of glucose-6-phosphate at the catalytic site of the enzyme.

Ischemia-Reperfusion Damage to Heart Tissue

Last year a collaborative study between researchers in the Laboratory of Biochemistry and the Surgery Branch of NHLBI was initiated to assess the biochemical changes which occur during ischemia-reperfusion in Langendorff hanging heart preparations. In an extension of these studies, it has been shown that during ischemia there is a marked loss of ATP, accompanied by rises in the levels of AMP, inosine, hypoxanthine and xanthine. There is also a decrease in the level of the heart-type lactic acid dehydrogenase isozyme and an increase in the liver-type isozyme. Upon reperfusion, all of these variables returned to normal values.

Section on Signal Transduction

Animals utilize a variety of chemicals to maintain an elaborate cell-to-cell communication network. These specific chemicals are synthesized and released by signaling cells and then move to other cells where they induce a specific response only in those target cells that have receptors for the extracellular signal molecules. On binding to their cell surface receptors, many extracellular signaling molecules including hormones, peptide growth factors, neurotransmitters and immunoglobulins, elicit intracellular responses by activating inositol phospholipid-specific phospholipase C (PLC). Activated PLC catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate diacylglycerol and inositol 1,4,5-trisphosphate (IP_3). Diacylglycerol is the physiological activator of protein kinase C (PKC) and IP_3 induces the release of Ca^{2+} from internal stores. This bifurcating pathway constitutes the cornerstone of a transmembrane signal transduction mechanism that is now known to regulate a large array of cellular processes, including metabolism, secretion, contraction, neural activity, and proliferation.

Direct protein isolation and molecular cloning studies have revealed the existence of multiple PLC isozymes in mammalian tissues. The various PLC isoforms appear to be activated by different receptors and different mechanisms and to interact differently with inhibitory mechanisms such as those mediated by cAMP-dependent protein kinase (PKA) and PKC. Our goal is to understand the molecular mechanisms by which the binding of receptor by extracellular signaling molecules triggers activation of PLC and the activity of PLC is regulated by PKC and PKA.

Ligation of T-cell antigen receptor (TCR) by antigens, lectins, or antibodies elicits a variety of early biochemical events that ultimately lead to cell proliferation and interleukin 2 production. Among the earliest biochemical events is the activation of PLC. We found that the TCR-dependent PLC activation is due to the phosphorylation of PLC-γ1 by unknown nonreceptor tyrosine kinase. We also observed that the extent of tyrosine phosphorylation of PLC-γ1 decreased when PKA or PKC phosphorylate PLC-γ1 at serine 1248, thus causing the reduction of PLC activity in T-cells treated with cAMP elevating agent or PKC activator. The 2 Fc receptors for IgG (FcγR), FcγRI, and FcγRII were also shown to be functionally coupled to a nonreceptor tyrosine kinase that phosphorylates PLC-γ1 after receptor cross-linking, thereby causing activation of PLC-γ1. Cross-linking of FcγRs on macrophages, monocytes and neutrophils activates a multitude of biological functions, such as antibody-dependent cellular cytotoxicity, phagocytosis, and release of inflammatory mediators. In addition to tyrosine phosphorylation-dependent activation, a variety of extracellular signals have been shown to regulate PLC activity through G proteins. The PLC-activating G protein has been identified to be G_q class. This $G\alpha_q$ specifically activated PLC-ß1 but not

PLC-γ1 and PLC-δ1. The $G\alpha_q$ family is comprised of $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, and $G\alpha_{16}$. We also cloned, sequenced and expressed a cDNA corresponding to a previously uncharacterized PLC-ß member. This new PLC was named PLC-ß2. Using the different members of $G\alpha_q$ and the 2 members of PLC-ß, we tested specificities. All 4 proteins of $G\alpha_q$ ($G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, and $G\alpha_{16}$) stimulated PLC-ß1 with $G\alpha_q$ and $G\alpha_{11}$ being most efficient. On the other hand, $G\alpha_{16}$ was most effective in activating PLC-ß2.

Section on Protein Function in Disease

Research in this section focusses on metal-catalyzed oxidative modification of proteins, a covalent modification which has been implicated in important physiologic and pathologic processes. These include the aging processes, arthritis, gene expression, hypertension, intracellular protein turnover, oxygen toxicity, and reperfusion injury after ischemia. Research goals include determination of the chemical and structural changes induced by oxidation; identification of the processes affected by oxidative modification; purification and characterization of the systems which catalyze the modification and subsequent proteolysis; understanding the controls which may regulate the modification and proteolysis of specific proteins; and application of this knowledge to the rational design of irreversible enzyme inhibitors.

In the last year, emphasis was placed on the establishment of collaborative investigations designed to identify processes in which oxidative modification of proteins occurred. Correlations were observed in peritoneal fluid of mice during tumorigenesis induced by chronic inflammation; in the plasma of rats after exposure to ionizing radiation; in the tracheal secretions of prematurely born humans who were supported by mechanical ventilation; in the proteins of cultured endothelial cells exposed to oxidative stress; and in the human protease inhibitor, alpha-2-macroglobulin, which was functionally inactivated by exposure to neutrophils.

These studies of oxidative modification *in vivo* require sensitive assays which can detect oxidative modifications in samples containing small amounts of protein. Several assays were developed for this purpose, all based on detected of the carbonyl group introduced into protein by oxidation. Work in progress aims to develop even more sensitive immunological assays which could be applied to very small samples, including biopsy specimens.

As an outgrowth of studies on targeted oxidative modification of proteins, it was found that low concentrations of copper were able to inactivate the protease from the human immunodeficiency virus. More detailed investigation of the inhibition established that it occurs at a site located on the surface of the enzyme, rather far from the active site. This finding suggests the possibility of developing a new class of protease inhibitors which are not directed to the substrate-binding site.

Section on Intermediary Metabolism and Bioenergetics

The importance of the trace element, selenium, in human and animal health can be attributed at present to its role in at least two essential enzymes, glutathione peroxidase and tetraiodothyronine-5´-deiodinase. The peroxidase is an important defense against organic peroxides that damage cell membranes and the deiodinase is essential for production of the active thyroid hormone from its inactive prohormone precursor. A deficiency in active thyroid hormone seriously limits normal growth and development processes. Failure to regulate the levels of lipid peroxides and other organic peroxides is believed to contribute to the incidence of heart disease and certain forms of cancer.

Studies with bacterial enzyme systems on the mechanism of biosynthesis of selenocysteine for specific insertion into selenium-dependent enzymes and the formation of 2-selenouridine residues in tRNAs from 2-thiouridine residues have shown that the highly reactive selenium donor used for these processes

is a new biological compound, selenophosphate. Selenophosphate is formed from selenide and ATP by selenophosphate synthetase in the overall reaction: $-SeH + ATP = SeP + Pi + AMP$. Selenophosphate was detected and shown to be identical with the synthetic reference compound by ^{31}P NMR spectroscopy. Catalysis of the exchange of AMP with ATP in the absence of selenide demonstrated that the first intermediate in the reaction sequence is an enzyme-pyrophosphate derivative. A specific cysteine residue in the enzyme, shown to be essential by site-directed mutagenesis, may be the group on the protein that interacts with selenide. A nearby lysine residue also is essential for catalysis of the overall reaction, but its replacement with glutamine does not prevent the AMP-ATP exchange reaction and therefore this lysine is not the pyrophosphate accepting group.

The gene encoding the selenoprotein A component of the glycine reductase complex was isolated from *Clostridium sticklandii*, cloned, sequenced, and expressed in *E. coli*. In studies on the biological role of selenoprotein A in the glycine reductase reaction it was shown that substrate levels of the pure protein could be reacted with [^{14}C]iodoacetate to form the same Se-carboxymethyl derivative that is produced from glycine by the complete enzyme complex. Reductive cleavage of this selenoether to form acetate is catalyzed by protein C in the presence of a reducing agent and arsenate. An acetylthiol ester derivative of protein C has been implicated as an intermediate in this second half reaction. Large-scale isolation of protein C and studies on the pure protein revealed that this enzyme component is remarkably stable. It withstands heating in SDS and this property has allowed separation of its two dissimilar subunits by SDS gel electrophoresis and reconstitution of active enzyme after recombination of the subunits.

In studies on the conversion of acetate to methane and carbon dioxide by the strictly anaerobic *Methanosarcina barkeri* the metabolic roles of a large number of vitamins and trace elements have been elucidated. The first intermediate, acetyl-CoA, involves pantothenate, a component of CoA. A large molecular weight enzyme complex catalyzes the conversion of acetyl-CoA to a methyl-B_{12} protein, carbon dioxide, and reduced Fe/S protein. The intermediate methyl group acceptor is a pterin, tetrahydrosarcinapterin (H_4SPT), which is converted to CH_3-H_4SPT. Carbon dioxide from the acetate carboxyl group is formed via CO by a nickel enzyme component. These intermediates have been shown by UV-visible and EPR spectroscopic analyses.

Section on Metabolic Regulation

The research projects of the investigators in this section are concerned mainly with studying the mechanism of enzyme action and its regulation.

Mechanism of Enzyme Action and Regulation

Protein ubiquitination. Protein ubiquitination has been implicated in numerous intracellular processes, such as protein turnover, DNA transcription, etc. The enzymes involved including the ubiquitin activating enzyme, E1, the ubiquitin carrier enzyme, E2, which transfers the activated ubiquitin to its ligase, E3, or directly ubiquitinates its target proteins. E1 was found to exist as two isoforms, designated as $E1_{117kDa}$ and $E1_{110kDa}$. It can be phosphorylated *in vivo* and *in vitro*. The later is catalyzed by protein kinase C. Two of the E2 isoforms, $E2_{32kDa}$ and $E2_{20\ kDa}$ are phosphorylated by a tyrosine kinase and a novel kinase in the cytosolic fraction of HeLa cells, respectively. Phosphorylation of E1 and $E2_{32kDa}$ resulted in doubling of their activities. Thus, it appears that protein ubiquitination is regulated by the phosphorylation/dephosphorylation mechanism. In addition, a role of ubiquitin in DNA transcription will likely be revealed by the finding that the transcription factors, fos and jun, undergo multiple ubiquitination.

E. coli ribonucleotide reductase. This enzyme consists of 2 substrate and effector-binding subunits, R1, and 2 tyrosyl radical and iron-containing subunits, R2. Kinetics and site-directed mutagenesis studies

revealed that the carboxyl terminus of the R2 subunit is the sole contributor in the R1-R2 subunit interaction and the conserved Y356 residue seems to serve as an intermediary in the electron transfer process.

Cytosolic Ca(II) oscillation. In response to external stimulant, many cells release Ca(II) in an oscillating manner. Initial studies on this mechanism revealed that hepatocytes and mesangial cells, upon vasopressin stimulation, produce inositol triphosphate with an initial spike and then maintain at an elevated steady-state level relative to that of basal. The results suggest that the frequency of Ca(II) oscillation, which is dependent on the concentration of agonist, is correlated with the steady-state level of inositol triphosphate.

Characterization of Mg(II)-dependent, Ca(II)-inhibited phosphatases in bovine brain. Three of these phosphatases have been identified. They are the 78,000 MW dimeric serine-threonine protein phosphatase with low specific activity, a 50,000 MW p-nitrophenylphosphate phosphatase, and a phosphoseryl protein phosphatase which consists of six 90,000 MW subunits. The latter has been purified to near homogeneity and partially characterized. Its activity is highly concentration-dependent, and it is activated by a 78,000 MW phosphatase and by polyamines.

EPR Study of Free Radicals in Biology

Cellular effects of elevated Cu,Zn-superoxide dismutase (Cu,Zn-SOD) activity. Previously, we showed that Cu,Zn-SOD catalyzes the conversion of H_2O_2 to $\dot{O}H$ radical which can generate secondary radicals from enzyme-bound anionic ligands or scavengers. Therefore, the enhanced activity can produce damaging effects to cells. In this study, NCB-20 cells were transfected with a human pSV_{neo} Cu,Zn-SOD gene expression vector. Formation of free radicals in these clones by H_2O_2 generated by a glucose/glucose oxidase system were monitored by EPR and spin trapping methods. The glutathionyl radicals formed in the transformed cells were half of that of the wild-type cells. These results indicate that the Cu,Zn-SOD overexpressed cells maintain chronic pro-oxidant states due to free radicals production catalyzed by the elevated level of Cu,Zn-SOD.

Mechanism of a protective enzyme against thiyl radicals. A protective protein purified and cloned by Rhee and coworkers is known to protect against oxidative modification by thiol-induced oxidants. Point mutation studies indicate that C47 is required for the protection activity. Thiyl radicals were produced and trapped by a spin-trap in solution containing oxygen, Fe(III), and dithiothreitol or reduced glutathione. Addition of wild-type or C170S modified protective protein quenches the spin adduct signals while C47S mutant does not. The results indicate the protective protein exerts its effect either by scavenging thiyl radicals directly or removing the sulphenyl hydroperoxide, produced through the reaction of thiyl radicals and oxygen.

Effect of Electric Fields on Biomembranes

When membrane of intact cells is subject to some critical electric field strength, they became transiently permeabilized. This transient time is inversely proportional to the ionic strength of the suspending medium. In addition, the ionic strength also affects the location of the permeabilization with respect to the electrodes, and the bulk-to-bulk membrane potential.

Section on Protein Chemistry

The Section on Protein Chemistry is studying the physical and chemical properties of macromolecules of biological interest and the roles of ligand binding and of protein-protein and inter- and intra-subunit interactions in enzyme catalysis and regulation. The energetics of ligand binding to proteins

involve contributions from both ligand-protein and protein-protein interactions. Ligand-promoted changes in protein-protein interactions underlie the phenomenon of cooperativity in ligand binding to proteins and, in addition, give rise to many examples of stabilization and destabilization of protein structures by ligands and metal ions.

A goal of studies on protein folding is to understand the stabilization of complex secondary and tertiary structures in native proteins that leads to their unique conformations. Assembly processes and biological interactions depend on the correct folding of polypeptide chains. If an unfolding reaction can be described as a reversible, two-state transition, the thermodynamics of protein folding/unfolding can be determined experimentally from measuring the free energy of unfolding as a function of temperature. Thermally induced reversible, partial unfolding transitions in dodecameric Mn•glutamine synthetase (622,000 M_r) from *E. coli* have been studied at pH 7 by using spectral techniques to monitor tryptophanyl and tyrosyl residue exposures and by differential scanning calorimetry (DSC) for direct measurements of thermodynamic parameters. Spectral results indicate that each enzyme subunit has two domains which unfold as the temperature is raised. DSC results show a single endotherm (centered at 51.6°C) and only two two-state transitions. Thus, cooperative interactions link the partial unfolding reactions of all subunits within the dodecamer. However, the overall enthalpy change is 176 $\pm$ 12 kcal(mol dodecamer)$^{-1}$ or only 1-4% of that estimated for complete unfolding. Effects of active-site ligands and protons on these unfolding processes have been quantitated. Thermally induced unfolding of *S. typhimurium* tryptophan synthase (143,000 M_r) also is being studied at pH 8 in order to elucidate the overall conformational stability of this $\alpha_2\beta_2$ multienzyme complex and to characterize intramolecular subunit interactions. DSC profiles for the holoenzyme (with bound pyridoxal phosphate) exhibit two well resolved endotherms corresponding to 2α and β_2 domains in the complex. Results indicate that: (1) thermally induced unfolding of the $\alpha\beta\beta\alpha$ complex is ~60% without significant disruption of α:β contacts; (2) the refolding processes are slowly reversible; (3) the coenzyme stabilizes α:β contacts as well as β_2; and (4) a pre-transition exposure of Trp residues in β chains occurs. In addition, recently obtained DSC profiles for phospho- and dephospho-myosin-II forms from *Acanthamoeba* show an identical, single sharp endotherm at ~42°C. The unfolding of the rod and head portions of myosin-II appear to be uncoupled by the presence of an ATP analogue.

High-affinity, sensitive metallochromic indicators have been used to characterize Zn^{2+} interactions with several biologically important proteins. Zn^{2+} has an essential role in maintaining the quaternary structure and thereby the allosteric properties of aspartate transcarbamoylase (ATCase) from *E. coli*. Zn^{2+} binding to structural sites in yeast arginase has been found to stabilize the trimeric structure, which is necessary for regulation of ornithine transcarbamoylase activity through multienzyme complex formation. Zn^{2+} also has a pivotal role in stabilizing the tertiary structure of transcriptional factor IIIA (TFIIIA) from *Xenopus laevis*, which is responsible together with at least two other factors (TFIIIB and TFIIIC) for directing the transcription of oocyte 5S RNA genes by RNA polymerase III.

Transcriptional factor IIIA (TFIIIA) from immature oocytes of *Xenopus laevis* has been specifically labeled at Cys-287 with a highly fluorescent probe (IAEDANS). DNA fragments from the internal control region (ICR) of the 5 *S* RNA gene also have been labeled at 4 specific positions with a fluorescein probe and purified. Fluorescence resonance energy transfer measurements on the 1:1 ICR DNA:TFIIIA complex are being made to obtain distances between probes and interaction constants. The wild-type and a mutant form (lacking ~100 residues at the C-terminal end) of TFIIIA also have been cloned.

ANNUAL REPORT OF THE
LABORATORY OF BIOPHYSICAL CHEMISTRY
SECTIONS ON CHEMICAL STRUCTURE
STRUCTURAL NUCLEAR MAGNETIC RESONANCE
AND BIOPHYSICAL INSTRUMENTATION
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

October 1, 1991 through September 30, 1992

This year has been characterized by several important changes in the Laboratory. Thus, there has been a conscious move by several of us to attempt to extend our techniques and expertise, largely developed with and for small molecules, to the increasingly important area of biopolymers including proteins, and oligosaccharides. The change of emphasis has been accompanied by delays in research due to the arrival of the 600-Mhz nuclear magnetic resonance spectrometer and the movement of a large part of the Laboratory to its new location.

The tools used by the Laboratory include mass spectrometry, nuclear magnetic resonance, x-ray crystallography, countercurrent chromatography, biocalorimetry,near infrared chromatography, and scanning tunneling and force field microscopy.

Acquisition of a 600 Mhz NMR system, along with other techniques, enables Ferretti and his group to determine the conformations of proteins in both the free and biologically critical bound states. With M. Nirenberg (NHLBI), his group is currently studying the 77 amino acid DNA binding domain of a regulatory protein coded by the homeobox gene from the fruit fly *Drosophila melanogaster*. The goal is to understand, by using the nuclear Overhauser effect, which intermolecular contacts are important for binding and regulation of the transcription process in general. He now has about 50% of the interatomic distances determined.

The same techniques are being applied to determine the interatomic distances, and thereby the conformation of the envelope glycoprotein (gp 120) of the AIDS retrovirus HIV-1. A 35 amino acid part of this protein is currently under study since it is the region most responsible for the virus' immunogenicity and therefore the development of vaccines. Complete data has been obtained and molecular modeling of the most probable structure is in progress.

The group is also continuing to study the Rift Valley Fever virus by employing a novel approach. They will determine the structures of eight ^{15}N octapeptides, each one the same except for the location of the label. These compounds will allow

unequivocal location of each nitrogen in the peptide. This octapeptide is the minimum responsible for antibody binding (epitope), and studies of its exact structure may be critical in vaccine development. This project is in an intermediate stage and is being accompanied by studies of the next larger peptide containing 14 more amino acids.

Critical to all of these studies is the ability to produce sufficient peptide for analysis by NMR,--whose sensitivity currently leaves much to be desired. For this reason Ferretti's group is examining the utility of *in vitro* protein synthesis (translation) using a computer for process control. They have recently successfully synthesized globin as a test protein and are now attempting to synthesize the major histocompatibility protein complex with bound peptide that is central to immunological studies.

In other NMR work, R. J. Highet has determined the structure of a modification of myoglobin brought about by its modification with bromotrichloromethane. L. Pohl (NHLBI) has used this reaction as a model for the destruction of cytochrome p-450 by xenobiotics such as halothane anesthesia gas and the antiinflammatory drug diclofenac in the liver.

The exact structure of compounds, including the interactions that take place between groups within the molecule, and their exact chirality (handedness) is critical to understanding their physiological activity. For these reasons, J. Silverton has determined the x-ray crystallographic structure of the anti-retroviral drug, adenalleen, and noted the presence of an unusual CS---HN bond. He has likewise determined the structures of two inhibitors of aromatase, the enzyme involved in steroid hormone biosynthesis, a phenylbutanediol used to examine hydroxylation in *Bovaria sulfurescens*, and a clathrate compound offering promise in the purification of drugs.

Direct visualization of biological structures is now possible using the new techniques of atomic force and tunnelling microscopy. This equipment has recently been acquired and experiments are in progress.

Y. Ito has been perfecting the technique of countercurrent chromatography for many years and has developed an apparatus that bears his name. It allows separation of samples of a wide variety of biologically important compounds at levels far surpassing those obtained by the now classical technique of high performance liquid chromatography (HPLC) and in addition there is no sample loss by irreversible adsorption. However, the quality of the separation is somewhat less than HPLC and its use with trace compounds is less straightforward. A second problem has been that because of its dependency on aqueous and organic

solvents, it is not easily applicable to proteins, a major interest in the laboratory. Ito has sought to overcome this deficiency by completely redesigning the apparatus and using polymeric liquid phases such as dextran and polyethylene glycol. As mentioned last year, he has achieved success with human serum albumin, the globulins, and histones. Now it has been extended to the separation of high- and low-density lipoproteins. Admittedly, its application to the separation of a minor biologically important protein from a complex mixture (in the fashion of HPLC) has yet to be demonstrated.

In a surprising development, Ito has discovered that the CCC technique has the ability to separate and purify multigram quantities of ionizable substances, concentrating the impurities in a very narrow zone. The technique takes advantage of very small differences in acidity and solubility to amplify differences in their separation. The method has been applied successfully to DNP amino acids and a variety of food colors that are regulated by the FDA for which very pure standards are required. It is considered that the technique should have wide application to drug purification.

R. Berger continues to explore the use of near infrared as a tool in the analysis of enzymatic reactions. He is now attempting to follow the hydrolysis of ATP with ATP-Ase in hopes of detecting intermediates in the reaction through their spectral signatures. In a second project, Berger has modified his differential microcalorimeter for titration -type measurements and used the apparatus to study micelle-monomer transitions and haemoglobin binding of carbon dioxide. The first study shows that the mechanism of micelle formation, a model of cell membrane structure, depends on the phospholipid used for its construction.

H. Fales has switched his interests largely to the mass spectrometry of biopolymers. He has recently examined the oligosaccharides involved in a series of demyelinating diseases and can now sequence them successfully, at least in terms of the types of sugars involved. In a second project, he has identified a compound involved in steroid biosynthesis as an isomeric form of adenosine diphosphate. He has analyzed a wide variety of peptides and several proteins involved in many different studies using the JEOL mass spectrometer operating with fast atom bombardment and electrospray sources. Ion trap mass spectrometry, with its promise of high sensitivity, mass range and resolution continues to be a major interest.

Annual Report of the Laboratory of Cardiac Energetics

National Heart, Lung and Blood Institute
October 1, 1991 through September 30, 1992

The major goal of the Laboratory of Cardiac Energetics is to improve understanding of the cellular and molecular processes involved in the conversion of energy to useful forms of work in the heart and other tissues. With this insight we hope to develop new strategies for the diagnosis, prevention and treatment of heart disease. Our technological approach to these problems is the use of non-invasive nuclear magnetic resonance (NMR) and optical spectroscopy techniques. These methods permit the non-invasive monitoring of several critical metabolites involved in cellular energy metabolism in both intact biological tissues and humans. The application of these technologies to humans allows us to evaluate these tools as non-invasive diagnostic modalities.

The major energy metabolism pathway we have been concentrating on over the last year is the complex interaction between energy conversion processes in the heart (i.e. oxidative phosphorylation), muscle contraction (i.e. pumping of blood) and coronary blood flow. For the heart to function properly, these three elements must be orchestrated with remarkable precision to provide the appropriate amount of oxygen, substrates and energy to support the pumping of the blood. Myocardial muscle contraction is believed to occur by the utilization of the energy in adenosine triphosphate (ATP), produced predominately by oxidative phosphorylation occurring in the mitochondria.

To use ATP for muscle contraction, ATP is hydrolyzed to adenosine diphosphate (ADP) and inorganic phosphate (Pi). It was long believed that these hydrolysis products of ATP served as the intracellular signals which regulated the rate of oxidative phosphorylation in the heart.

While the further breakdown of ADP to adenosine was believed to regulate the resistance of the coronary blood vessels and thereby control the coronary blood flow. However, as we have previously demonstrated, the hydrolysis products of ATP, ADP and Pi do not change during physiological increases in work. That is, even under conditions where the turnover of ATP increases almost 5-fold, the hydrolysis products of this reaction do not increase. This indicates that the ATP, ADP and Pi levels are highly buffered by oxidative phosphorylation in the healthy myocardium and that these metabolites are unlikely to play an important role in the orchestration of metabolism or coronary blood flow. Therefore, some other cytosolic parameters must be responsible for the orchestration of these critical processes in the heart. Over the last year we have evaluated new sites where these regulatory processes could be occurring in the regulation of coronary blood flow and metabolism during work stress.

With regard to the metabolic regulation of blood flow, previously we established the role of the so-called "ATP-sensitive" K channel (ATP-K) in the regulation of coronary resistance in the intact heart in vitro and in vivo. The ATP-K channel is inhibited by intracellular ATP concentrations on the order of 100 uM and was believed to remain closed during the normal function of the heart, where the ATP concentration approaches 6 Mm. However, recent studies have shown that this channel is modulated by other metabolites, including lactate, ADP and hydrogen ions. In addition, this channel is critical for the acute increase in blood flow which occurs with ischemia or hypoxia.

We used a specific inhibitor of this channel, glibenclamide, to evaluate the effects of channel closing on coronary vascular resistance. We found that glibenclamide increased the vascular resistance of the heart, resulting in functional ischemia as evidenced by an increase in lactate production and a decrease in tissue oxygenation (based on the optical spectra of myoglobin and cytochrome) in isolated perfused rabbit hearts and in vivo canine hearts. Thus, an open ATP-K channel is apparently present in the intact heart and is critical in the maintenance of coronary vascular tone. These data indicate that the ATP-K channel is open under normal conditions in the intact heart and that this channel may play a critical role in the regulation of coronary blood flow.

The hypothesis for how the ATP-K channel influences the coronary vascular tone is that the ATP-K channels hyperpolarize the smooth muscle plasma membranes, inhibiting Ca entry through the potential-sensitive Ca channel. Thus, according to this model, an open ATP-K channel vasodilates the coronary arteries by inhibiting Ca entry into the vascular smooth muscle.

We tested this hypothesis by evaluating the effects of inhibiting the potential-sensitive Ca channels on the ATP-K channel effects on vascular tone. Verapamil, an inhibitor of the potential-sensitive Ca channels and a strong vasodilator, completely inhibited the vasoconstrictor effects of gliburide. Another vasodilator, nitroprusside, which does not directly affect the potential-sensitive Ca channels, had no effect on the gliburide vasoconstriction. The data are consistent with the hypothesis that the ATP-K channel is affecting coronary vascular tone via membrane potential effects on the potential-sensitive Ca channel.

A major area of current interest in the laboratory is to establish whether the channel is dynamically modulated by metabolic events in the cell and the possible intracellular transduction mechanisms which may modify the activity of this channel and, therefore, coronary vascular tone.

Previous studies on the control of oxidative phosphorylation in the intact heart suggested that the utilization of substrates may be a

rate-limiting step. Indeed, we had proposed that the level of mitochondrial NADH, generated by substrate oxidation, may be a key controlling step in the regulation of oxidative phosphorylation. To evaluate this hypothesis, we investigated the effects of work on the mitochondrial NAD redox state. These studies demonstrated that the NAD redox state is constant as a function of work and oxygen consumption despite the fact that the flux of reducing equivalents through this system has more than doubled.

These results demonstrate that the NAD redox state is highly regulated and buffered in the intact cell, in contrast to results in isolated mitochondria. Furthermore, these results show that the level of NADH cannot be primarily responsible for the increase in oxidative phosphorylation rate with work since its level is remaining constant. Thus, our original hypothesis is apparently incorrect: Despite the fact that the NADH redox state is regulated, it apparently is not solely responsible for the stimulation of oxidative phosphorylation occurring with increases in work. We have begun to evaluate other possible control sites in the mitochondrial oxidative phosphorylation, including the role of the F1-ATPase in metabolic regulation.

Lactate and pyruvate metabolism of the intact heart has been evaluated in both in vitro and in vivo preparations. We became interested in lactate and pyruvate utilization after reports in the literature suggested that the overall metabolic consequences of these substrates were very different, even though they differ only by one metabolic reaction, lactate dehydrogenase. Dr. M. Laughlin, using a combination of 13C and 31P NMR on an in vivo canine heart preparation, established that pyruvate is the "preferred" substrate between these two metabolites, with pyruvate actually inhibiting net lactate uptake.

In addition, pyruvate was found to increase the phosphorylation potential in the heart in vivo, making more potential energy available for work. This again suggests that substrate supply is rate-limiting for the metabolic capacity of the heart even in vivo. In contrast, lactate metabolism did not result in any alteration of the phosphorylation potential in vivo. This is surprising since, as mentioned above, lactate and pyruvate only differ by one dehydrogenase reaction.

To investigate the possibility that the mitochondrial NADH redox state may be the source of some of these differences, Dr. F. Heineman measured the mitochondrial redox state in isolated perfused hearts using optical spectroscopy techniques. Both lactate and pyruvate were equally effective in increasing the concentration of mitochondrial NADH in the intact heart. Thus, the differential effects of lactate and pyruvate could not be explained by a change in mitochondrial redox potential.

In the course of these studies, it was noticed that lactate would specifically increase the concentration of a phosphate metabolite in the 31P NMR spectrum of the heart. Pyruvate did not increase the concentration of this metabolite. The chemical shift of this metabolite in the intact heart and in extracts suggests that it is a sugar phosphate, possibly glucose-6-phosphate. However, the precise identification and physiological significance of this metabolite has yet to be established.

Our working hypothesis for the differences in lactate and pyruvate metabolism effects in the intact heart involves the role of the cytosolic NADH redox state influencing the cytosolic phosphorylation potential, or free energy available to perform work, and the levels of intermediary metabolites which may also influence numerous metabolic pathways. This is currently being investigated by directly monitoring the cytosolic redox state and evaluating the specific cytosolic compounds generated by lactate or pyruvate metabolism.

A large portion of our efforts over the last year have been devoted to the development of NMR techniques to the study of organ physiology and biochemical structure in vivo. These approaches are being developed to provide new tools in studying the function of the body in vivo, as well as potentially providing new non-invasive diagnostic tools to clinically evaluate humans.

Using a saturation transfer approach, in combination with standard magnetic resonance imaging (MRI) techniques, we have successfully imaged the rate of magnetization transfer between various macromolecules, protons and protons in water in intact tissues. The contrast generated by this process, termed magnetization transfer contrast (MTC), is unique in magnetic resonance imaging and is currently being developed for clinical applications around the world. These applications include the study of multiple sclerosis, blood vessel angiography, cancer and sports medicine. This approach has also provided a unique insight into the basic mechanisms of water proton relaxation in biological tissues which we have been concentrating on over the last year.

In our attempts to evaluate the molecular mechanisms responsible for the MTC effect, we have studied a large series of macromolecules and lipids. In summary, these studies have demonstrated that a surface hydroxyl group is the most effective surface group in generating this effect, while amine groups can provide a weak effect. A combination of temperature, magnetic field dependence, Ph and isotope effects have led us to a unique model of this interaction.

Isotope and pH studies demonstrated that chemical exchange with the hydroxyl group is not rate-limiting for the MTC effect. The temperature dependence of this process is very complex. At room to

physiological temperatures, there is little or no temperature dependence, while at temperatures from 0 to 25 degrees C there is a strong positive dependence on temperature. At high temperatures (25-50 degrees C) there is a negative temperature dependency. We interpret these data to represent the interaction of two processes, the low temperature data revealing a site restriction for water access to the surface reaction sites, and the high temperature effects relating to the correlation time of the water macromolecule complex. The combination of these two effects around room temperature results in the apparent lack of temperature dependence in this temperature range.

Our current working hypothesis for this magnetic interaction of water with the macromolecules involves an electric dipolar interaction of water with a surface hydroxyl group. This electric dipolar interaction is much stronger than in free water due to the low electric dipole potential in the lipid interface, resulting in the required long residence times of water in the sites (usec). The water, oriented by its interaction with the hydroxyl group, then undergoes a magnetic dipolar interaction with a backbone proton in close proximity to the hydroxyl group, resulting in the magnetization exchange. We are currently testing this model with the use of site-specific isotope replacements in macromolecule models. We are also further evaluating the frequency and temperature dependency of this process.

Since November the Laboratory has been outfitting a 4 Tesla 1-meter bore NMR system for human studies. This is the highest magnetic field strength available for whole-body NMR studies. Only 2 similar systems exist in the world. We have demonstrated that this high-field system will improve the signal-to-noise ratio in spectroscopic studies of tissue biochemistry as much as 3-fold, as well as improving the spatial and time resolution of NMR imaging studies as much as 3-fold and 7-fold, respectively. These advantages will make possible many new human studies which could not be performed at the more commonly available fields of 1.5 or 2 Tesla.

The magnet was constructed by Oxford Magnetics, while much of the other hardware was provided by General Electric. We have successfully added a research console which permits us to program state-of-the-art NMR acquisition schemes in this prototype system. In addition, the entire front end of the system has been rebuilt and is currently operating at state-of-the-art signal-to-noise and rf performance. Several studies have been initiated, including 31P NMR studies of human skeletal and cardiac tissue, magnetic field susceptibility imaging, 13C NMR studies of human glycogen metabolism and MRI microscopy of the human body.

A major 4T project led by Dr. R. Turner is the use of MRI techniques in the evaluation of tissue oxygen levels. These studies

deal with the development of methods for non-invasive monitoring of perfusion and blood oxygenation, and real-time imaging. The basic experimental tool in these studies is Echo-Planar Imaging (EPI), a type of magnetic resonance imaging which acquires images in 0.1 sec. or less -- much faster than conventional MRI. The images can be sensitized to spatial variations in blood flow and blood oxygenation and can be obtained at a rate of up to 1 per second.

In the past year, we have mainly been engaged in studies of blood oxygenation in cat brain and functional activity in human brain. At a magnetic field of 2T, we have used a cat respiratory challenge model to observe the time course of imbalances of oxygen supply and utilization in brain tissue, and using the Laboratory's new whole-body 4T magnet, we have performed experiments studying the localization of visual and motor function in the brains of human volunteers. The following findings have been made: 1) for constant blood volume, there is a linear relationship between the change in relaxation rate observed in gradient-echo MR images of cat brain and the change in blood hemoglobin saturation caused by respiratory challenges; and 2) active regions in primary visual and motor cortex in human volunteers can be identified using gradient-recalled echo-planar images. Photic stimulation results in changes of image intensity in highly focal regions in the primary visual cortex of up to 25 percent.

The determination of blood deoxyhemoglobin content is based on the different magnetic properties of oxygenated and deoxygenated red blood cells. Deoxygenated blood is paramagnetic. This creates a strong magnetic field susceptibility effect around deoxygenated red blood cells. This effect can be imaged and quantitated using magnetic resonance imaging techniques which are sensitive to these effects. Using this approach, Dr. Turner has been validating the blood oxygen tension measurement in animal models and applying this technique to functional imaging of the human brain. In the animal studies, Dr. Turner has shown, using EPI sequences, that there is apparently a linear relationship between the concentration of deoxyhemoglobin and the MRI signal, and that this technique can be used to evaluate the kinetics of ischemia nad reperfusion oxygenation in the brain and kidney. In humans, Dr. Turner has demonstrated that active regions in the primary visual nad motor cortex can be identified using EPI during stimulation of these regions using photic stimulation or hand movement. This has provided a technique of unparalleled spatial and time resolution to map the metabolic functional responses of the brain to stimuli.

Annual Report of the Laboratory of Cell Biology
National Heart, Lung, and Blood Institute
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This year the Section on Molecular Cell Biology was created with Dr. John A. Hammer, III as Section Head. The Laboratory comprises nine independent investigators (Drs. Korn, Hammer, Hendler, Greene, Eisenberg, Bowers, Kolobow, Knustson and Chen) and two emeritus scientists (Drs. Bowman and Flavin) who are still active in research. The Laboratory occupies approximately 9800 square feet in Building 3 and 2000 square feet in Building 10 with use of animal surgery space in Building 14E. Major research interests continue to be: (1) the structure and function of non-muscle myosins, (2) the regulation of actin polymerization, (3) the structure and function of the 70-kDa heat shock proteins, (4) bioenergetics, (5) biological applications of fluorescence spectroscopy, (6) respiratory assist devices, and (7) membrane flow in eukaryotic cells.

Structure and Function of Non-muscle Myosins: There are two independent programs: Dr. Edward Korn investigates the myosins of the oil amoebae, *Acanthamoeba castellanii*, while Dr. John Hammer investigates the myosins of *Dictyostelium discoideum*. Previous work in this Laboratory had shown the existence of two classes of myosins in amoebae - the conventional myosin II and the novel myosin I. These studies led to the demonstration by other investigators of myosins I in many other species including mammals and other vertebrates. Indeed they initiated a revolution in the thinking about myosins, the consequent discovery of yet other classes, and the realization of the existence of a myosin superfamily.

Myosins II have two heavy chains and two pairs of light chains. Each heavy chain has a globular head (with an ATPase site and a site that binds F-actin which activates the ATPase activity) and a tail (that forms a coiled-coiled alpha-helix with the other tail through which the myosins self-associate into bipolar filaments). The light chains are associated with the heads near the head-tail junction. The actomyosin ATPase activity is regulated in different ways in myosins II from different sources. *Acanthamoeba* myosin II is inactivated by phosphorylation of up to three sites near the C-terminal tail of each heavy chain approximately 90 nm from the globular head. A major interest of Dr. Korn has been to understand the mechanism of this regulation.

Previous work had led to the proposal that regulation occurred through an induced conformational change in the structure of the filament, i.e. each molecule in the filament would have the same activity irrespective of the level of its own phosphorylation but dependent on the overall phosphorylation state of the filament as a whole. It was proposed that this could happen because the structure of the filament was such that the phosphorylation sites of all of the monomeric subunits were clustered near the hinge region in the heavy chains. A change in phosphorylation state then might alter the conformation of the hinge such that the globular heads on the projecting arms would interact differently with F-actin.

Electric birefringence studies in collaboration with Dr. Donald Rau, NIDDK, have now provided the first evidence for a structural difference between phosphorylated and dephosphorylated filamentous myosin II (they are

indistinguishable by electron microscopy, circular dichroism, and sedimentation analysis). The electric field induces a dipole in the otherwise symmetrical molecules which leads to birefringence. The signal is very much greater for phosphorylated than for dephosphorylated myosin II. Thus the dephosphorylated myosin II appears to be much less flexible around the hinge region.

The proposed regulation mechanism predicts that the ATPase activity of monomeric myosin should be unaffected by its phosphorylation. This has now been demonstrated in experiments in which the myosin was maintained in a monomeric state by adsorption onto nitrocellulose-coated capillary pipettes before filling the pipettes with the assay mixture that would otherwise have induced the myosin to form filaments. Dephosphorylated and phosphorylated monomers had the same high activity as dephosphorylated filaments and only phosphorylated filaments were inactive.

However, phosphorylated and dephosphorylated monomers are not functionally identical as the former, despite its high ATPase activity, is inactive in an *in vitro* motility assay (as are phosphorylated filaments) while the latter is as active as dephosphorylated filaments. In addition, both monomeric and filamentous dephosphorylated myosin are much more rapidly cleaved within the globular head than are monomeric and filamentous phosphorylated myosin by endoproteinase Arg-C. Both are cleaved at two sites - Arg 638 and Lys 621 - but phosphorylation greatly suppresses the rate of cleavage at Arg 638. Thus, somehow the effects of phosphorylation at the tip of the tail appear to be projected through the coiled-coil alpha helix and influence both *in vitro* motility activity and proteolytic susceptibility. Among other things, these results show that the long tails of myosins II have functional importance other than to serve as the basis for formation of bipolar filaments.

Acanthamoeba myosins I have a single heavy chain and one or two light chains. The heavy chain has a globular head homologous to that of myosins II but a short, non-helical, non-filamentous tail that does not support self-assembly. The short tail contains a membrane-binding site and a second actin-binding site. Dr. Korn's group showed previously that the ATPase activity of actomyosin I is greatly stimulated by phosphorylation of a single residue in the head, that the kinase that phosphorylates the heavy chain is activated by autophosphorylation and that the autophosphorylation is greatly accelerated by acidic phospholipids.

Work this year has shown (1) that the *Acanthamoeba* myosins I bind to and their autophosphorylation is stimulated by plasma membranes *in vitro*, (2) that the membrane-bound kinase can phosphorylate membrane-bound myosin I and (3) that the ATPase activity of the membrane-bound myosin is activated by this phosphorylation. Thus, the presumed physiologic regulatory system has been re-created *in vitro*. Moreover, Ca^{2+}-calmodulin has been found to regulate the system. The heavy chain kinase binds to calmodulin in the presence of Ca^{2+} and Ca^{2+}-calmodulin competes with phospholipid and inhibits the phospholipid-activation of kinase autophosphorylation. Moreover, Ca^{2+}-calmodulin also inhibits the phosphorylation of myosin I by the kinase. Thus, acting through calmodulin, Ca^{2+} ultimately inhibits actomyosin I ATPase activity. In general, Ca^{2+} has the opposite effect on myosins II which it activates by different mechanisms depending on the source of the myosin II.

On the assumption that this redundancy might be most pronounced within, if not limited to, the members of each sub-family, the first approach was to prepare a double mutant in which antisense DMID was expressed in DMIB$^-$ cells. This has succeeded in producing a stable mutant that expresses none of the DMIB isoform and less than 0.5% of the DMID isoform expressed by wild type. The phenotype of this mutant, however, differs very little from the phenotype of the single DMIB$^-$ mutant. Therefore, the next step will be to prepare a triple mutant in which neither DMIB, DMID nor DMIC (the third known isoform in this structural sub-family) is expressed.

Regulation of Actin Polymerization: Actin, a major component of the cytoskeleton, is a 42,000-Da protein that polymerizes into long helical filaments. The polymerization process and the organization of the filaments are highly regulated within cells by the interaction of actin monomers and polymers with many different actin proteins. Polymerization occurs through an initial formation of trimers followed by the rapid elongation of these nuclei. A protein, actobindin, that appears to regulate the critical nucleation step was discovered several years ago in this Laboratory by Dr. Korn. This small (88 amino acids) protein was shown to bind two actin monomers *in vitro*, consistent with its sequence which shows a 33-residue repeat.
This year, a number of experiments were carried out to determine whether the actual ligand for actobindin might be the actin dimer that would be the first intermediate in the polymerization process. As a model system, two different groups of covalently cross-linked dimers were prepared with a variety of cross-linkers under previously established conditions. Cross-linking the subunits of F-actin produces, among other products, a so-called upper dimer while cross-linking actin intermediates formed early in the polymerization process produces lower dimers (named by their relative electrophoretic mobilities).

Actobindin had a greater affinity for the cross-linked upper dimers than calculated from its affinity for two actin monomers but its affinity for the cross-linked lower dimers was about the same as for two monomers. However, when actin was cross-linked under polymerizing conditions in the presence of enough actobindin to inhibit polymerization, only lower dimer was formed. One interpretation of these data is that the upper dimer has the conformation of the normal polymerization intermediate with which actobindin interacts and that this interaction converts the upper dimer to the lower dimer which is incapable of polymerization and accumulates. This would allow actobindin to act catalytically rather than stoichiometrically which is the experimental observation.

70-kDa Heat Shock Proteins: When cells are subjected to an increase in temperature a number of proteins, collectively called heat-shock proteins, are produced. The 70-kDa family is one of the most prominent set of heat-shock proteins in all species; some of them are essential to cell viability and are constitutive while others are produced only during heat shock. They appear to function constitutively as "molecular chaperons" involved in the folding and unfolding of other proteins and in protein translocation processes and also to protect other proteins against denaturation. They all bind ATP tightly. One of the first constitutive 70-kDa heat shock protein characterized is the uncoating ATPase (UC ATPase) that *in vitro* catalyzes the ATP-dependent removal of clathrin from coated vesicles.

Drs. Evan Eisenberg and Lois Greene have been studying the relationship between the ATPase and uncoating activities of bovine brain UC ATPase, the role of the bound nucleotide in the binding of substrates to the UC ATPase and the possibility that the mammalian UC ATPase might substitute for 70-kDa heat shock proteins in yeast as an approach to understanding their function *in vivo*.

Previously, Greene and Eisenberg proposed a reaction scheme in which (1) 3 molecules of UC ATPase bound to each clathrin triskelion on a coated vesicle, (2) hydrolysis of the ATPase-bound ATP to ATPase-bound ADP and Pi accompanied release of the clathrin as a UC ATPase complex, and (3) replacement of the ADP and Pi with bound ATP caused the dissociation of the free clathrin from the UC ATPase which (4) could then interact with another coated vesicle. The model predicts that 3 ATP molecules would be hydrolyzed per clathrin triskelion released but this could not be shown because of the high ATPase background in the preparations.

To circumvent this problem, clathrin baskets were assembled *in vitro* from purified clathrin and a mixture of clathrin assembly proteins. Although ATP hydrolysis was associated with the disassembly of the baskets the mixture of clathrin and assembly proteins stimulated UC ATPase activity in the absence of baskets. Several individual purified assembly proteins were then tried. With clathrin baskets formed from clathrin and purified assembly protein AP180, Eisenberg and Greene were able to demonstrate a stoichiometric initial burst of ATPase activity accompanying the stoichiometric initial burst of basket disassembly as their theory predicts.

During the preparation of purified assembly proteins, it was found that assembly protein AP2 could not be used because it stimulated a burst of ATPase activity in the absence of clathrin. It has now been possible to separate two factors from the AP2 preparation. One of these greatly stimulates an initial burst of hydrolysis of ATP when added to UC ATPase with bound ATP. The second factor is required for uncoating to occur when baskets are assembled from clathrin and pure AP2.

The 70-kDa proteins interact with a number of different substrates, e.g. UC ATPase interacts with a peptide C and pigeon cytochrome C peptide in addition to clathrin and at the same site. In ATP, clathrin binds to UC ATPase about 10 times more tightly than the two peptides. In ADP, there is a modest increase in affinity of clathrin for UC ATPase but a very great decrease in the rates of attachment and detachment. ADP increases the affinities of the two peptides about 100 times, however, but again with a reduction in the on and off rates. Thus, a major effect of the bound nucleotide may be to regulate the rates of attachment and detachment of ligands to and from the UC ATPase. Surprisingly, nucleotide-free UC ATPase behaves as UC ATPase with bound ATP. Thus, the regulation appears to be through the bound ADP.

Yeast has 8 70-kDa proteins of which 4 resemble the bovine brain UC ATPase. Yeast strains are available that produce none of these 4 proteins but remain viable because one is supplied by a transforming plasmid dependent on a galactose promoter. Thus, the yeast is viable when grown in galactose media but not in glucose media. Insertion of a multicopy plasmid containing the UC ATPase transformed the yeast into a strain that could grow on glucose as expected if the UC ATPase were expressed and able to substitute for the yeast

70-kDa protein that was now not expressed. However, it has thus far been impossible to verify that the mammalian UC ATPase is expressed in the yeast when grown on glucose.

Bioenergetics: The main objective of this research under the direction of Dr. Richard Hendler is to understand the mechanism of conversion of the energy derived from the flow of electrons during oxidative metabolism into metabolically useable energy such as ATP or an electrochemical gradient across a membrane that can drive transport processes. Dr. Hendler has developed the methodologies to establish voltage control of the system so that the intermediates in the process at various equilibrium positions can be identified and the energy with which they bind electrons determined. A kinetic approach allows definition of the reaction sequences and the mechanisms by which electrons are passed from one intermediate to another.

Cytochrome oxidase is a critical component of the respiratory path; electrons flow from cytochrome c to cytochrome oxidase and then to O_2. The energy released in this process is converted into a proton electrochemical gradient that can then be used to do work or synthesize ATP. Cytochrome oxidase is believed to have 4 or 5 redox centers: heme $\underline{a}$, heme $\underline{a}_3$, Cu_A, Cu_B and possibly a Cu_X. The sequence of electron transfer within the cytochrome oxidase complex has been a major focus of research in Dr. Hendler's laboratory. The traditional view has become that each heme has only one redox center, that the first electron is bound to either heme but then, through negative cooperativity, the second electron is bound at lower potential. The electron flow is thought to be from cytochrome c to heme $\underline{a}$ to heme $\underline{a}_3$.

Dr. Hendler had previously reported a total of four redox potentials for the two hemes (i.e. two for each heme): the lowest and highest potentials are associated with heme $\underline{a}_3$ and the two intermediate potentials with heme $\underline{a}$. In a collaboration with Dr. Ira Levin, NIDDK, utilizing resonance Raman spectroscopy, Dr. Hendler has confirmed the existence of the four redox centers and their attribution to the respective hemes. In addition, another redox potential for heme $\underline{a}_3$ was discovered.

Furthermore, utilizing this information and the new ultrarapid spectrometer that has been developed by Dr. Hendler and collaborators in BEIP and DCRT, Dr. Hendler has obtained data that suggest that the flow of electrons from cytochrome c is to heme $\underline{a}_3$ and then to heme $\underline{a}$ rather than the reverse as is the current belief.

Biological Applications of Fluorescence Spectroscopy: The program directed by Dr. Jay Knutson has the principal objective of developing new fluorescence spectroscopic techniques, particularly time-resolved spectrofluorometry, for application to biomedical questions. The research includes designing and building spectrofluorometers with new capabilities, developing new data analysis methods and demonstrating the applicability of these new techniques to problems of protein, DNA and membrane structure that cannot be solved by other methods. The time-resolved system monitors the conformational changes in structure by examining the changes in fluorescence of tryptophan and tyrosine residues.

This year particular attention was devoted to protein-DNA interactions. The tryptophan and tyrosine fluorescences of the polypeptides are quenched or

enhanced by DNA binding. Studies have been initiated with three enhancers of transcription - TFIIIA, HSF and oct-pou - and three enzymes that act on DNA - BAM HI, beta-polymerase and HIV integrase.

Dr. Raymond Chen has applied conventional and time-resolved fluorescence methods to the study of several systems. Human basic fibroblast growth factor binds tightly to the cell receptor only when the bFGF is complexed with heparin or a heparan glycoprotein. The anti-tumor drug Suramin interferes with tight receptor binding. Fluorescence studies, coupled with circular dichroism, showed that heparin and Suramin cause different conformational changes in bFGF by close binding to the receptor-binding loop of bFGF, i.e. within 12.5 angstroms of the tryptophan in the binding loop.

Alpha-lactalbumin is an interesting protein that modifies the activity of galactose synthetase. In the absence of lactalbumin, the enzyme preferentially transfers galactose from UDP-galactose to N-acetylglucosamine residues of proteoglycans. Lactalbumin inhibits this reaction and facilitates transfer of galactose to glucose to synthesize lactose. Xray crystallography shows that lactalbumin has a structure similar to that of lysozyme but the polysaccharide-binding cleft that is functional in lysozyme is blocked in lactalbumin by tyrosine 103. A new fluorescence species occurs in lactalbumin during a thermal transition at 35-37° C involving tyrosine 103 suggesting that conformers of lactalbumin may be formed that can bind saccharides.

The techniques developed by Dr. Knutson have recently been used to detect both fluorescent and absorbent objects in tissue. In tissue models it has been possible to detect displacements of less than 1/50th of an inch of absorbing objects. As color as well as fluorescence can be observed this method may become applicable to quantifying oxygenation state, pH, electrolytes, etc, as well as objects such as tumors within tissues.

Respiratory Assist Devices: Mechanical pulmonary ventilation at peak inspiratory pressures is frequently used to maintain ventilation in diseased lungs. Dr. Theodor Kolobow had previously shown that similar treatment of healthy sheep frequently leads to adverse reactions that are histologically indistinguishable from acute respiratory failure that can lead to multiorgan system failure. This is thought to be caused by overdistension of the normal lung.

To avoid these problems, Dr. Kolobow has devised an intratracheal pulmonary ventilation system which delivers air at small tidal volumes to local areas of the lung - 10-20% of healthy tissue is sufficient to allow eventual weaning to room air ventilation. This year, an ultrathin walled, non-kinking spring wire reinforced endotracheal tube was developed with the shape modified so that it can adapt to the shape of the larynx of the subject. The resistance of this tube to airflow is equal to or less than that found in healthy humans. It is hoped that this device will reduce the morbidity that now accompanies pulmonary ventilation.

Previous work on the *in situ* localization of the three myosin I isoform and the myosin I heavy chain kinase in *Acanthamoeba* have been extended by both immunofluorescence and quantitative immunoelectron microscopy. The three isoforms are distributed very differently. Myosin IA is mostly in the actin-rich cell cortex just below the plasma membrane with a small amount in patches on the plasma membrane and associated with small cytoplasmic vesicles. Myosin IB is mostly on the plasma membrane where it is generally uniformly distributed but enriched at regions of pseudopod extension and on large cytoplasmic vacuoles. Myosin IC is distributed in patches on the plasma membrane, on large cytoplasmic vacuoles and uniquely on the contractile vacuole membrane. Kinase is largely cytoplasmic but also on the plasma membrane.

This leads to the proposal that myosin IA functions mainly to move filaments in the cortex relative to one another and to move small cytoplasmic vesicles to and from the cell surface. Myosin I would function mostly in moving plasma membrane relative to the cell cortex and at the leading edge of cells as well as moving large vacuoles to and from the surface. Myosin IC might share some of the roles of myosin IB and also have a unique function in the contractile vacuole.

The different localizations are supported by the observation that the three myosin I isoforms and kinase bind to different sub-populations within a purified plasma membrane preparation *in vitro*. Thus, targeting to membrane sites apparently depends on different docking proteins in the membranes.

Dictyostelium has at last five myosin I isoforms and possibly four more by DNA analysis. Dr. Hammer has been studying four of these that have been best characterized DMIA, DMIB, DMID and DMIE. DMIB and DMID are very similar to the three *Acanthamoeba* isoforms in that they have the globular head common to all myosins and a short tail with putative membrane-binding and actin-binding sites. DMIA and DMIE have shorter tails that probably are missing the second actin-binding site but still have the membrane-binding site. Thus, the tail domains define two sub-families; the incompletely sequenced DMIC probably is in the same sub-family as DMIB and DMID. The existence of two sub-classes was supported this year by showing that the expression of DMIB and DMID increases about 3-7-fold during chemotactic aggregation while expression of DMIA and DMIE is unaffected.

Immunofluorescence localizations by Dr. Hammer using isoform specific antibodies, has shown that both DMIB and DMID are at the leading edges of migrating and dividing cells, at the phagocytic cups and at sites of cell-cell contact - a distribution very similar to that of *Acanthamoeba* myosin IB. In contrast, preliminary results with antibodies against DMIA show diffuse cytoplasmic staining and no staining at the leading edge or at cell-cell contacts.

Dr. Hammer has used genetic approaches to determine the function of the individual *Dictyostelium* myosin I isoforms. DMIB$^-$ cells show impaired rates of ameboid movement (50% of wild type), abnormal pseudopod formation, slower aggregation rates, a longer doubling time and a reduction (35%) in phagocytic rate. All these are consistent with the localization of this isoform but the relative mildness of the effects suggests redundancy of functions among the various myosin I isoforms.

ANNUAL REPORT OF
THE LABORATORY OF CELLULAR METABOLISM
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

October 1, 1991 through September 30, 1992

Research in the Laboratory of Cellular Metabolism has been for several years concentrated on guanine nucleotide-binding proteins that are critical control elements and signal transducers in cellular responses to the extracellular environment. Many of these are substrates for bacterial toxins that catalyze the ADP-ribosylation of specific amino acids and thereby alter their activity. The properties and actions of some of these toxins have also been investigated, along with continuing attempts to identify and characterize analagous ADP-ribosyltransferases in animal cells, as well as the enzymes that could remove the ADP-ribose and the proteins that are substrates in these kinds of potentially regulatory ADP-ribosylation cycles. Long term studies of specific cyclic nucleotide phosphodiesterases have yielded recently considerable new information about a family of these enzymes that are important sites of hormonal effects in many cells and are targets of several new drugs. Much of our effort in the past year has been focussed on the 20-kDa so-called ADP-ribosylation factors (ARFs) that are involved in intracellular vesicular transport to Golgi membranes and perhaps in other locations. This has included cloning and expression to evaluate structure-function relationships and effects of post-translational modification as well as attempts to define interactions of individual specific ARFs with Golgi in vitro.

1. Cyclic Nucleotide Phosphodiesterases

These enzymes that degrade cAMP and cGMP are of diverse types with different and complex regulatory properties to provide for many-facetted and precise control, both temporal and spatial, of concentrations of these critical messengers. We had purified and characterized several of these enzymes in the past but most recently efforts have been focussed on the so-called cGMP-inhibited phosphodiesterases that are implicated in the regulation of vital processes in many cells, e.g., lipolysis in adipocytes, aggregation of platelets, contractility of cardiac myocytes, relaxation of vascular smooth muscle cells. Our early studies were of the fat cell enzyme, which was referred to as the hormone-sensitive cAMP phosphodiesterase, identified because its activity was increased by exposure of the cells to insulin. We later established that the antilipolytic action of insulin results from its activation of this phosphodiesterase. This phosphodiesterase is also inhibited rather selectively by cilostamide. A related inhibitor was incorporated into an affinity matrix that we used to purify the adipose tissue enzyme.

Last year we cloned from rat adipose tissue cDNA libraries two different cGMP-inhibited phosphodiesterases. One (RFAT2) is very similar in nucleotide and deduced amino acid sequences to a human cardiac cGMP-inhibited phosphodiesterase that we recently cloned. The other cDNA (RFAT 1) hybridized with an mRNA that is present predominantly in adipose tissue. Neither hybridized very strongly with RNA from liver or brain. Both cDNAs were incorporated into expression vectors to yield glutathione-S-transferase fusion

proteins that were synthesized in E. coli. The RFAT 1 fusion protein was inhibited by cGMP and cilostamide. It reacted more strongly than did the RFAT 2 fusion protein with antibodies against the bovine adipose tissue cGMP-inhibited phosphodiesterase, whereas antibodies against the human platelet enzyme reacted more strongly with the RFAT 2 fusion protein. Using the RFAT 1 cDNA, this mRNA was detected predominantly in fat cells and the appearance of RFAT 1 mRNA correlated with differentiation of 3T3-L1 into adipocytes.

The deduced amino acid sequence of RFAT 1 includes at least three domains. A putative membrane association domain in the N-terminal region (amino acids 73 to 251) is of interest as the hormone-sensitive adipocyte phosphodiesterase is isolated in microsomal fraction. In the putative regulatory domain (amino acids 252 to 695), there are three consensus A-kinase phosphorylation sites (RRXS), consistent with the fact that the adipose enzyme is activated by hormones that elevate cAMP, as well as by insulin. In collaborative studies we found that one of these sites is phosphorylated by A kinase in vitro. Although the putative catalytic domain (amino acids 696 to 980) is clearly related to those of other phosphodiesterases, there is an insertion of 44 amino acids (740 to 783) that does not align with other phosphodiesterases. There are insertions of 44 different amino acids in the deduced sequences of RFAT 2 and HCAR 2 consistent with view that this region is specific to the cGMP-inhibited phosphodiesterases and may be important in the identification, localization, expression, or regulation of individual members of the family. The adipocyte and human cardiac cGMP-inhibited phosphodiesterases have little similarity in amino acid sequences except in the catalytic domains. They have, however, very similar hydropathy index plots and may well have similar three dimensional structures and functional domains.

RFAT 2, which hybridized predominantly with mRNA from rat heart and also rat adipose tissue but not from rat fat cells, was used to screen another rat adipose tissue cDNA library. One positive clone (~7.1 kb) was >90% identical in nucleotide sequence to a human cardiac cGMP-inhibited phosphodiesterase clone that we had isolated earlier. Whereas the deduced sequences of the human and rat cardiac enzymes are very similar in the regulatory domains the adipocyte and cardiac enzymes are not. The human cardiac and adipocyte genes are, in addition, located on different chromosomes. Although all of these four phosphodiesterases have extensive similarities in catalytic domains, the so-called "additional region" of 44 amino acids clearly distinguishes cardiac and adipocyte enzymes and may be useful for identifying other potential members of the cGMP-inhibited phosphodiesterase family.

Based on information from the additional region, oligonucleotides were synthesized and used to screen human aortic and liver cDNA libraries. Several different clones from both libraries had nucleotide sequences identical with that of the human cardiac cDNA, but a possibly unique cGMP-inhibited phosphodiesterase sequence has been cloned from the human aortic cDNA.

To determine whether protein kinase C (C Kinase) isozymes modulate cell cAMP levels via effects on phosphodiseterases, cultured NIH 3T3 cells that express only the α isozyme were used. Total phosphodiesterase activity was increased as much as five times in cells transfected with γ C kinase. Treatment of these cells with an activator of C kinase (PMA) decreased phosphodiesterase activity (~75% in 15 min; $t_{0.1}$ = 8 min), whereas it had no effect on control cells or on cells transfected with β C kinase.

To identify which phosphodiesterase(s) was affected by transfection with γ C kinase, phosphodiesterase activity was assayed under different condition and with selective inhibitors. In control and β C kinase-transfected cells,

the predominant (>90%) phosphodiesterase was a Rolipram-sensitive type IV enzyme. A Ca^{2+}- and calmodulin-sensitive enzyme accounted for the rest of the activity. In the γ C Kinase-transfected cells, the latter seemed unchanged, but about 30% of the total activity was due to a cGMP-inhibited enzyme and the Rolipram-inhibited phosphodiesterase was only about 60%. These observations were confirmed by FPLC separation of phosphodiesterases on DEAE-5 PW. Experiments in progress are designed to identify which phosphodiesterase is decreased in activity by PMA treatment of cells transfected with γ C kinase. This demonstration of an interaction between the action of a specific phosphodiiesterase and a specific C kinase isozyme establishes a physiologically significant functional difference among C kinase isozymes and should provide new clues to mechanisms of interaction (or intersection) of cAMP and C kinase regulatory pathways.

2) ADP-ribosylation Factors (ARF): 20-kDa Guanine Nucleotide-Binding Proteins

ARF was first identified in Gilman's laboratory as a GTP-binding protein required for the cholera toxin-catalyzed ADP-ribosylation of purified $G_{s\alpha}$. We later showed that ARF enhances all of the toxin catalytic activities, independent of ADP ribose acceptor and that there is a family of mammalian ARF proteins, of which we have now cloned and expressed six. Each of them enhanced cholera toxin ADP-ribosyltransferase activity in a GTP-dependent fashion, although as found last year, the requirements for activity of recombinant human ARF 6 and bovine ARF 2 synthesized in E. coli were distinctly different. This is probably true also for at least some of the other ARFs and is reflected in the differences noted earlier in requirements for stability and activity of ARF purified from different tissues.

It was somewhat surprising to find that recombinant ARF 6 synthesized in E. coli as a fusion protein with maltose-binding protein was active without added GTP. This was explained when the nucleotide bound to the ARF 6 fusion protein was identified as GTP by HPLC, whereas GDP was bound to non-fusion rARF6 (and is bound to ARF purified from animal tissues). Removal of bound nucleotide from the fusion protein by dialysis against urea resulted in completely GTP-dependent activity. It appears that in the fusion protein the bound GTP is protected from hydrolysis (or release in the absence of urea treatment), consistent with the general view that these processes require the interaction of ARF with GTPase-activating proteins and/or guanine nucleotide-exchange proteins.

There are two ARF proteins in Saccharomyces cerevisiae, which are 96% identical and are essential for cell viability. The yeast ARF 1 gene is constitutively expressed, whereas the ARF 2 gene is repressed by glucose. Expression of human ARF 5 or ARF 6 or Giarda ARF was able to rescue the lethal ARF double mutant, although the rescued strains grew much more slowly than did wild-type yeast or strains rescued with yeast ARF 1. We infer that these heterologous ARFs, which are only ~60% identical to yeast ARFs, do not function very efficiently or effectively in the yeast protein secretory system, presumably because they lack one or more structural elements that provide for the requisite specificity of protein-protein interactions involved in guanine nucleotide exchange and/or membrane targeting.

There is increasing evidence that ARFs have a physiological role in the vesicular transport of proteins from endoplasmic reticulum to Golgi (and perhaps in endocytosis), moving back and forth from cytosolic to membrane locations. In tissue homogenates, ARF is largely cytosolic. On addition of GTP or a non-hydrolyzable analogue, ARF accumulates in membrane fractions, or

associates with added phospholipid. ARF, unlike many other small GTP binding proteins (e.g., ras), is not modified at the C-terminus by isoprenylation and/or palmitoylation. It is co-translationally modified by myristoylation of the N-terminal glycine exposed after removal of the initiating methionine. To assess the role of N-terminal myristoylation in GTP-dependent ARF binding to membranes, recombinant human ARF 5 was synthesized in E. coli with and without co-expression of yeast myristoyl CoA: protein N-myristoyltransferase. Myristoylated and non-myristoylated rARF 5 partially purified from bacterial cultures were incubated with a brain Golgi fraction for 40 minutes before Golgi was pelleted and Golgi-bound ARF activity was assessed by measuring activation of cholera toxin ADP-ribosyltransferase activity. Only myristoylated ARF incubated in the presence of GTPγS at 37°C was bound to Golgi, indicating that myristoylation is necessary for this association, but is not sufficient, as myristoylated ARF with GDPβS did not bind. Myristoylation did not significantly alter GTP binding by ARF or its ability to activate cholera toxin. It appears that myristoylation is important for ARF association with membranes, although specific binding likely requires interaction with a target protein.

Microsequencing of sARF I and II, that we had purified earlier from bovine brain, established that they are ARF 1 and 3, respectively. Rabbit antibodies (IgG) against sARF II reacted similarly with ARFs 1, 2, and 3 (class I) on Western blots. ARF 1 and 3 were distinguished by their electrophoretic mobilities. Antiserum against rARF 5 cross-reacted partially with rARF 4, but not detectably with rARF 6 and minimally with class I ARFs. GTPγS increased recovery of ARF activity and immunoreactivity in organelle fractions separated by density gradient centrifugation, after incubation of rat brain homogenate with ATP and a regenerating system. ARF I accumulated in microsomes plus Golgi and Golgi fractions, whereas ARF 5 seemed to localize more specifically in Golgi. The smaller increment in ARF 3, which was overall more abundant than ARF 1, was distributed more evenly among fractions. On incubation of Golgi with a crude ARF fraction from brain, GTPγS, and an ATP-regenerating system, association of ARF activity with Golgi increased with increasing ATP concentration paralleled by increases in immunoreactive ARF 1 and 5, and to a lesser degree ARF 3. Golgi incubated with GTPγS and purified ARF 1 or 3, bound more ARF 1 than ARF 3. Based on immunoreactivity and assay of ARF activity, individual ARFs 1, 3 and 5 appeared to behave independently and selectively in their GTP-dependent association with Golgi <u>in vitro</u>.

Last year, we described the genes for human ARFs 1 and 3 and noted that the locations of intron splicing sites in the coding regions of those genes and the bovine ARF 2 gene are identical. Completion of characterization of the ARF 2 gene this year, revealed that it does differ from the other class I ARF genes (ARFs 1, 2, and 3) in that it has an inverted CCAAT box in an appropriate location and exhibits a selectivity of tissue and species expression not observed for any other mammalian ARF. It appears to be developmentally regulated in rat brain. Potential regulatory sequences in the promoter region of the gene and in the 3' UTR of the mRNA may provide clues to the basis for the pattern of ARF 2 expression.

E. coli heat labile enterotoxin (LT), which is involved in the pathogenesis of travelers' diarrhea, resembles cholera toxin structurally, and like CT, catalyzes the ADP-ribosylation of $G_{s\alpha}$ as well as several model substrates. It is also activated by ARF in a GTP-dependent manner. Cholera toxin (CT) and <u>E. coli</u> heat-labile enterotoxins (LT) are oligomeric proteins composed of B or binding subunits linked to an A subunit that possesses ADP-ribosyltransferase activity. The enzymatic activity of the A

subunit is latent. Its expression requires trypsinization in a domain near the carboxy terminus that links the A1 and A2 proteins and reduction of the single disulfide bond linking A1 and A2. The reduced and alkylated A1 protein was enzymatically active and stimulated by ARF. These data are consistent with a model in which ARF interacts with the activated form of toxin. To determine whether forms of toxin with latent activity were also capable of interaction with ARF, we assessed the effects of proteolysis and reduction on generation of an ARF binding site. Replacement of Glu 112 of the LT A subunit with Lys (E112K) resulted in a catalytically inactive toxin (Tsuji et al., (1990) J. Biol. Chem. 265, 22520). The ability of this mutant LT (E112K) to inhibit ARF stimulation of ADP-ribosylation catalyzed by reduced and alkylated CT A1 protein was examined. Activation of LT requires both trypsinization and reduction to generate LTA1. The requirements for inhibition of rhARF 6-stimulated activity by LT(E112K) were identical to those needed to generate an active A1 protein, suggesting that the appearance of an ARF binding site coincides with appearance of an active catalytic site. When the concentration of ARF is limiting, an enzymatically inactive protein containing an ARF binding site could serve as a competitive inhibitor of the active toxin. The fact that basal ADP-ribosylagmatine formation was inhibited suggests that toxin-toxin interactions may also be a factor in expression of ADP-ribosyltransferase activity.

3) ADP-ribosylation of Proteins in Animal Cells.

The presence in animal cells of NAD:arginine ADP-ribosyltransferases was first demonstrated in this laboratory. These enzymes catalyze the same model reaction as does cholera toxin and in cells, presumably catalyze the transfer of ADP-ribose from NAD to specific arginines in their protein substrates, which remain to be identified. ADP-ribosylarginine hydrolases that catalyze the removal of ADP-ribose from the modified protein would obviously be necessary to complete an intracellular ADP-ribosylation cycle with a potential for regulatory importance. Such activities were identified and, with antibodies generated against a hydrolase purified from rat brain, immunoreactive 39-kDa hydrolase proteins were demonstrated in several tissues. Last year, a hydrolase clone was isolated from a rat brain cDNA library and expressed in E. coli. Subsequently cloned human and mouse ADP-ribosylarginine hydrolases are >79% identical to it in deduced amino acid sequences. Repeated attempts over a considerable time to isolate cDNA clones for a mammalian NAD:arginine ADP-ribosyltransferase may finally be successful, if a clone obtained this year yields an expressed protein that exhibits the expected activity.

Last year, while looking for a cysteine-specific ADP-ribosyltransferase in animal cells, we found that free cysteine reacted non-enzymatically with free ADP-ribose to form a product identified as ADP-ribosethiazolidine carboxylic acid with a chemical stability that distinguishes it from ADP-ribosylcysteine. In the course of those studies, incubation of brain fractions with radiolabelled ADP-ribose resulted in labelling of several proteins, including some with stability like that of ADP-ribosylcysteine. One of these, after purification and microsequencing, was identified as aldehyde dehydrogenase, an enzyme with a catalytic cysteine in the active site. In relatively brief assays with purified aldehyde dehydrogenase, ADP-ribose was a competitive inhibitor with respect to NAD. After longer incubation or with higher concentrations of ADP-ribose, inhibition was irreversible. Covalent modification increased to 2 mol per mol of enzyme with increasing ADP-ribose

concentration or time, as enzyme activity decreased to <10% of control ($t_{0.5}$ = 2.7 h), consistent with half-sites reactivity postulated for the tetrameric enzyme. NAD effectively decreased the covalent modification and inactivation caused by ADP-ribose, whereas acetaldehyde was much less protective. Several other enzymes, with and without active site cysteines, were modified by ADP-ribose, but to less than 10% the extent of modification of aldehyde dehydrogenase. It is clear that reactivities of cysteines in different proteins differ greatly. Whether this kind of non-enzymaatic ADP-ribosylation of proteins occurs in cells is unknown. Perhaps it does in specific sites where ADP-ribose is generated in close proximity to a particularly reactive cysteine.

Annual Report of the Laboratory of Chemical Pharmacology
October 1, 1991 to September 30, 1992

During the past few years, this Laboratory has focused on studies related to drug-induced hypersensitivity reactions and other mechanisms of adverse responses to drugs and other environmental chemicals. 1) The Section of Enzyme Drug Interaction studies factors that govern the substrate specificity and product formation by the various cytochrome P-450 enzymes. 2 The Section on Pharmacological Chemistry studies the mechanisms by which chemically reactive metabolites, such as that formed from halothane, are synthesized in cells and form neoantigens. 3) The Section on Cellular Pharmacology focuses attention on mechanisms by which antigens cause the release of inflammatory mediators from mast cells. 4) the Section on Drug Tissue Interaction focuses on the development of methods for studying drug dependence by morphine analogs and for detecting the expression of messenger RNA for tissue specific isoforms of various enzymes including adenyl cyclase. A small group devotes its interest to mechanisms of dopamine uptake and release and their prenatal and postnatal development. In addition, the Sections have collaborated on studies of the mechanisms of drug-induced irreversible alterations of hemoproteins.

Mechanisms of Release of Mediators from Mast Cells

The activation of mast cells is responsible for immediate hypersensitivity reactions and probably underlies the chronic inflammatory component that ensues from some of these reactions: for example, airway hypersensitivity in asthma. Our objective is to delineate the signalling pathways for each of the various responses of these cells to antigen challenge. These responses include; secretion of granules with the immediate discharge of histamine and other mediators; generation of arachidonic acid and its metabolites; and the induction of synthesis of a variety of cytokines, such as tumor necrosis factor (TNF_{α}), which help recruit other inflammatory cells to the initial site of antigen challenge. The model for our studies is the cultured rat RBL-2H3 cell which, like the mast cell, contain receptors for immunoglobulin E (IgE). These cells are activated through multivalent binding of antigen to receptor-bound IgE, thereby inducing aggregation of the receptors within the plasma membrane, and resulting in the activation of phospholipase C to generate inositol 1,4,5-trisphosphate and diglycerides which in turn mobilize Ca^{2+} from intracellular stores and activate protein kinase C. Presumably the latter two events provide the necessary synergistic signals for secretion. Activation of phosphatidylcholine-specific phospholipase D provides another substantial source of diglycerides in antigen-stimulated cells. Our present focus is to define how these early biochemical events lead to the rapid extrusion of secretory granules and the delayed production of cytokines, as exemplified by TNF_{α}. We are also studying the mechanisms by which influx of external Ca^{2+} (an absolute requirement for secretion of granules) is promoted in RBL-2H3 cells.

This year we have elucidated the roles of individual isozymes of protein kinase C in RBL-2H3 cells. We previously had found that phosphorylation of myosin light and heavy chains by protein kinase C was correlated with secretion and that protein kinase C transduced both positive (for secretion) and negative (suppression of phospholipase C activation) signals in RBL-2H3 cells. We now

find that RBL-2H3 cells contained Ca^{2+}-dependent (α and β) and Ca^{2+}-independent, (δ, ϵ and ζ) isozymes of protein kinase C. In addition the washing of permeabilized cells removed all of the isozymes and resulted in a complete loss of secretory response to antigen but did not alter the phosphinositide response. By adding back small amounts (nM) of purified recombinant preparations of the isozymes, we have found that the secretory response to antigen was mediated primarily by the β and to a lesser extent by the δ isozymes - other isozymes were virtually inactive - and the inhibitory feed-back regulation of phospholipase C was mediated exclusively by the α and the ϵ isozymes. Exocytosis, however, required elevated $[Ca^{2+}]_i$, i.e. > 200 nM, whereas feed-back regulation occurred at basal $[Ca^{2+}]_i$, i.e. <100 nM.

Previous studies of signal transduction mechanisms through other receptors, either native (i.e. receptors for adenosine) or foreign (i.e. transfected muscarinic m1 receptors) to the RBL-2H3 cells have revealed that these receptors mediate the same array of signals via phospholipase C as does the IgE receptor, but utilize different mechanisms for the activation of phospholipase C either through G-proteins or tyrosine kinases. We have now shown that stimulants of all these receptors mobilize Ca^{2+} from the same intracellular pool of Ca^{2+} in the absence of extracellular Ca^{2+}. Influx of Ca^{2+} occurs through the same, or very similar, pathways as indicated by competition for uptake with various multivalent cations. Blockade of re-uptake of Ca^{2+} into inositol 1,4,5-trisphosphate-sensitive Ca^{2+} stores by thapsigargen or uptake into mitochondrial pools by mitochondrial inhibitors showed that external $^{45}Ca^{2+}$ equilibrated with the trisphosphate-sensitive pool in unstimulated cells and with mitochondrial pools of high storage capacity in stimulated cells. The findings support a simple dynamic model in which influx is a permissive process that allows, 1) refilling of the trisphosphate-sensitive pools and 2) at elevated $[Ca^{2+}]_i$, entry into mitochondrial pool. The regulatory point for Ca^{2+}-mobilization was the rate of discharge of Ca^{2+} from the inositol 1,4,5-trisphosphate-sensitive pools. The model provides an explanation for the past difficulty in demonstrating activation of discrete Ca^{2+}-conducting channels in activated mast cells.

Other highlights this year include the finding (with R.I. Adelstein) that the light chain of myosin is phosphorylated by the Ca^{2+}-regulated light chain myosin kinase at two sites (serine-18 and threonine-19). The serine 18 site was already phosphorylated in unstimulated cells but the threonine-19 site was only phosphorylated during the course of antigen-stimulation. The latter phosphorylation as well as the previously mentioned phosphorylation by protein kinase C correlated with the kinetics of secretion. Indirect evidence with various kinase inhibitors suggested that both phosphorylations may be necessary for secretion. Thus exclusive roles for Ca^{2+} and protein kinase C appear to operate for both secretion and myosin phosphorylation in antigen-stimulated cells. Also production and release of TNF_α were found to be distinct separate processes, both of which required continued aggregation of receptors (i.e. they could be aborted by displacement of antigen with monovalent hapten), elevation of $[Ca^{2+}]_i$ and activation of protein kinase C. TNF_α was not incorporated into secretory granules and was presumably secreted by some other mechanism. Finally, a novel 100 kDa GTP-binding protein has been discovered in RBL-2H3 cells. This protein, which is present in many tissues, is associated with the endosomal fraction, but can be detached with micromolar quantities of GTP. Determination

of the amino acid sequence of the N-terminus of the protein and identification of an additional sequence that is common to all α-subunits of G proteins (the receptor-binding domain) have allowed us to proceed with cloning of the gene (Ronit Sagi-Eisenberg).

Identification of Neoantigens Produced by Halothane, its Analogs and Other Drugs

In a few patients (about 1:35000) the anesthetic gas, halothane ($CF_3CHClBr$), causes a severe and frequently lethal hepatic necrosis, presumably by a hypersensitivity reaction. Despite the rarity of the toxicity, its occurrence has severely limited the use of halothane in many countries even though halothane has many beneficial qualities. Patients suffering from halothane-induced liver necrosis produce antibodies that react with several hepatic proteins in animals treated with halothane. These findings suggest the possibility that the necrosis might be caused by reactions of one or more of the antibodies with altered proteins on the surface of hepatocytes. During the past few years the Laboratory has identified all of the major neoantigens. Depending on the human sera used, the antibodies recognize trifluoroacetylated derivatives of GRP 94 (100 kDa), GRP 78 (82 kDa), ERp 72 (80 kDa), carboxylesterase (59 kDa), protein disulfide isomerase (57 kDa) and a P-450 (54 kDa). We also have succeeded in forming the trifluoroacetylated derivatives of many of these neoantigens in primary tissue cultures of mouse and rat hepatocytes. In addition, we have found that pre-treatment of the tissue cultures with tunicamycin; which blocks glycosylation, causes improper folding of proteins, and stimulates synthesis of certain proteins; increases the accumulation of several of the neoantigens by several fold. We hope that such pretreatments will enhance our changes of developing an animal model for halothane hepatotoxicity. Last year we reported that other halogenated ethanes, including those suggested as replacements for freon as a propellant and refrigerant, undergo metabolic activation to form trifluoroacetyl halides that form the same neoantigens as halothane. This kind of activation occurs with CFC-123 (CF_3CHCl_2), HCFC-124 ($CF_3CHCFCl$), and HCFC-125 (CF_3CHF_2). During the past year, we found that at the same dosages, HCFC-123 trifluoroacetylated the neoantigens to about the same extent as halothane, but that HCFC-124 trifluoroacetylated them about 10% as rapidly and HCFC-125 about 2% as rapidly as HCFC-123. These findings not only confirmed theoretical calculations that introduction of fluorine decreases the rate of hydrogen abstraction, but also suggested that HCFC-124 and HCFC-125 might be safer replacements of freon.

During the studies of the carboxylesterase as a neoantigen, we developed a specific antibody that we have used histochemically to identify organs that have unusually large amounts of the enzyme. This year, we found that the enzyme was present in nasal olfactory epithelium and thus may be responsible for the nasal toxicity produced by many esters found in the environment, such as ethyl acrylate, dimethyl glutamate, dimethyl succinate and ethyl adipate.

Several nonsteroidal antiinflammatory agents cause idiosyncratic hepatitis as manifested by hemolytic anemia, agranulocytosis and kidney damage. Last year we developed an antibody against one of these drugs, diclofenac, and have found that rats convert the drug to a metabolite that becomes covalently bound to a 110 kDa

protein in liver. This year we have used the antibody in histochemical studies and have found that the covalently bound metabolite is localized in the bile canalicular domain of the plasma membrane of hepatocytes. The identity of the 110 kDa protein and its possible role in the toxicity caused by nonsteroidal inflammatory agents remains to be established.

Mechanisms of Metabolism-based Alterations of Hemoproteins

Although many chemically reactive metabolites are sufficiently long-lived to escape the enzyme that catalyzes their formation and to become covalently bound to many other proteins and other macromolecules, some chemically reactive metabolites are short-lived and never leave the active site of the enzyme. Since many chemically reactive metabolites are produced by hemoproteins including cytochrome P-450 enzymes, we studied mechanisms by which chemically reactive metabolites might alter various hemoproteins. For example, various alkenes and halogenated methanes may produce free radicals that: 1) combine with heme in the hemoprotein, 2) combine with amino acid moieties in the active site of the hemoproteins, 3) induce the formation of heme radicals that combine with the protein. Our studies revealed that bromotrichloromethane ($BrCCl_3$) and hydrogen peroxide cause the covalent binding of heme to the protein of reduced myoglobin and hemoglobin. Moreover the alteration shifted myoglobin from an oxygen storage protein to an oxidase, which during redox cycling forms superoxide and hydrogen peroxide and leads to the further destruction of myoglobin. It was also determined that in the altered myoglobin the α carbon of heme's I vinyl was attached to histidine 93 of myoglobin and the β carbon contained a CCl_2 group. During the past year, computer models have been simulated with the CHARMM program and compared with the known crystal structure of native myoglobin. The simulations indicated that the heme in the modified myoglobin is only partially embedded in the protein, which permits water to enter the active site and the ferrous oxygen complex to undergo autoxidation to form superoxide anion radical and hydrogen peroxide. (Osawa)

During the past year, we extended our studies to 3,5-dicarboxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine (DDEP), a porphyrinogenic agent that destroys the activity of several forms of cytochrome P-450 through the activation of the ethyl group. Our studies of the deuterium isotope effects suggest that the DDEP is activated by a one electron abstraction from the nitrogen. The mechanism by which DDEP inactivates P-450 enzyme varies with the form. Cytochromes P-450 2CII and P-450 2C6 are inactivated by ethylation of the heme, whereas cytochrome P-450 3A is inactivated by covalent binding of the heme to the apoprotein. These findings thus explain why cytochromes P-450 2CII and 2C6, but not 3A, can be reactivated by addition of native heme. (Sugiyama) Some of these reactions can be mimicked by a system consisting of horseradish peroxidase, DDEP and either H_2O_2 or iodosobenzene. In this system, the altered, reversibly bound heme contains additional ethyl and hydroxyl groups.

Actions of Nitric Oxide

Nitric oxide, derived from arginine, nitroglycerin and amyl nitrites, is known to activate the hemoprotein, guanylate cyclase. During the past year we have found that nitric oxide mediates the release of dopamine from slices of striatum

evoked by N-methyl-D-aspartate, a postsynaptic glutamate receptor agonist (Hanbauer), and inhibits cytochrome P-450 enzymes both reversibly and irreversibly.(Osawa)

Mechanisms of Cytochrome P-450 Enzymes

Unlike most enzymes, many cytochrome P-450 enzymes form several metabolites from the same substrate. But several mechanisms may be envisioned by which the different metabolites may be formed. The mechanisms we have considered may be placed in two different groups. In the nondissociative group, the substrate reacts with the cytochrome P-450 to form (ES) complexes with different orientations. These complexes are then activated to dioxygenated complexes (EO_2S), which undergo dehydration to mono-oxygenated complexes (EOS). The (EOS) complexes may or may not be interconvertible but the substrate never dissociates from the (EOS) complexes into the medium. Instead, they must lead to the formation of the metabolites or be reduced to (ES) complexes and water. In the dissociative mechanisms the (EOS) complexes are formed in the same way, but do dissociate to (EO) and S and then may be reformed either in the same or different orientations but they are not directly interconvertible. Inspection of the equations derived for these mechanisms revealed that the dissociative and the nondissociative mechanisms may be most easily and usually unequivocally distinguished by comparison of the deuterium isotope effects on the (Vmax/Km) for the metabolite formed from the isotope insensitive pathway in noncompetitive experiments with the ratio of the rates of formation of the metabolite in 1:1 mixtures of the deuterated and nondeuterated substrates. In the nondissociative mechanisms, the isotope effects in the competitive experiment will be the same as those in the noncompetitive experiments. But in the dissociative mechanism the isotope effects in the competitive experiment, will always be 1.0.

Studies of the metabolism of testosterone and [2,2,4,6,6-$^2H_5$]-testosterone by cytochrome P-450 2CII revealed that the 2α-hydroxytestosterone and 16α-hydroxytestosterone (16α-OHT) are formed predominantly by a dissociative mechanism, because the isotope effects on (Vmax/Km) for the formation of 16α-OHT was inverse, whereas in the competitive experiment it approached 1.0.

Other Studies

Dopamine Uptake: The molecular mechanisms by which dopamine is released and taken up in presynaptic neurons are incompletely understood. In the past we found that in primary cultures of mesencephalic neurons from rat embryos, dopamine uptake and binding sites of its inhibitors appear simultaneously and increase proportionately during maintenance of the cultures. However, the binding sites of dopamine uptake inhibitors are in the cytosol rather than in the cell membranes. Moreover, the binding sites of the inhibitors in striatal synaptosomes prepared from prenatal rats are also in the cytoplasm. After birth, however, there is a translocation of the binding sites; at six days after the birth the binding sites appear to be located only in the neuronal membranes. During the past year, we found that the binding sites in the cytosol of prenatal rat brain and in the cultures are not associated with the dopamine transporter, but appear to be associated with low affinity piperazine-binding sites.

Endogenous Modulator of L-type Calcium Channels: Several years ago Hanbauer isolated a modulator of L-type calcium channels from rat brain. But its identity and mechanism remains unknown. Previous work established that it inhibits nitrendipine binding; markedly enhances L-type calcium current in cardiac and portal vein myocytes; and either increases or decreases $[Ca^{2+}]_i$, depending on the tissue. This year she found that the modulator causes a suppressant action in myocytes treated with cAMP. Moreover, the modulator also decreased the calcium current in myocytes treated with phorbol-12,13-diacetate, even though this treatment did not affect isoproterenol-induced increases in the calcium current. Thus the modulator does not act through the same mechanism as isoproterenol.

Effects of Morphine on cAMP in Human Neuroblastoma Cells: Morphine causes a dose-dependent decrease in cAMP formation induced by either prostaglandins or forskolin alone or in combination with ionomycin. As suggested by others, these effects plausibly could be the initial events in the development of physical dependence of morphine and its analogs. In support of this view withdrawal of morphine and some of its analogs after prolonged pretreatment of these cell s resulted in supersensitivity to forskolin-induced cAMP formation. Only those analogs that are known to produce physical dependence in animals produced hypersensitivity to forskolin-induced cAMP formation. For example, viminol, Z4349 and Z4381 induce supersensitivity but Z4666 and Z4669 do not.

Use of Polymerase Chain Reaction (PCR) Techniques in Identifying the Expression of Isoforms of Guanylate Cyclase in Human Tissues: Humans possess at least three different forms of guanylate cyclase, but the form(s) actually expressed in different organs is obscure. Because guanylate cyclase in human retina, liver and pancreas is stimulated by the arterial naturetic factor, it seemed likely that guanylate cyclase A was expressed in these organs. But confirmation of this view has been difficult. For this reason cDNA was formed using the mRNA from human retina and liver and the resulting cDNAs amplified by the PCR using oligonucleotides that were specific for guanylate cyclase A. Sequencing of the resulting PCR product confirmed that guanylate cyclase A was expressed in retina and liver. Further confirmation was obtained by Northern blots using probes derived from the PCR products.

Calcium Binding Domains of Calmodulin: Calmodulin is known to contain four domains that bind with calcium and a cationic carbocyanin dye, called "Stains-all". Nine polypeptides representing each of domains and several variants have been synthesized and the circular dichroism (CD) characteristics of their complexes with Stains-all studied. The complexes formed from all of the domains showed ellipticity of the J band in the CD spectrum which could be decreased by the addition of calcium. The dye had the highest affinity for domain IV and the lowest affinity for domain I. Moreover, replacement of aspartic acid by asparagine in the domain I abolished the binding of the dye, indicating that aspartic acid plays an important role in the binding with Ca^{2+} to domain I. A similar substitution in domain IV decreased the ellipticity of the J band, whereas replacement of tyrosine by tryptophan increased it. Such experiments thus provide clearer insights into the mechanism of Ca^{2+}-binding to calmodulin.

ANNUAL REPORT OF THE
LABORATORY OF KIDNEY AND ELECTROLYTE METABOLISM
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1991 to September 30, 1992

Our continuing goal is to analyze the function of the kidney as a basis for understanding its pathophysiology and treating its disorders. Since the formation of urine depends upon the transport of water and solutes by kidney tubules, understanding renal function requires analysis of these cellular processes and of their integration in the kidney. Therefore, we are studying transport by cells in general and kidney cells in particular, as well as the mechanisms, hormonal and other, that support and control transport and metabolism.

Isolated segments of renal tubules

In order to understand kidneys at a cellular and molecular level the functions of the different types of epithelial cells must be identified. Progress in this direction has relied heavily on the direct study of individual nephron segments. Each nephron segment has a different cell morphology and function. An important method (which originated in this laboratory) for directly studying the nephron segments is to dissect them and perfuse them individually in vitro. The findings during the past year using this method are as follows:

Maeda and Knepper are investigating hormonal signalling in the inner medullary collecting duct (IMCD) with the ultimate goal of learning how transport of salt, urea, and water are regulated. Using measurements of hormone-induced cyclic AMP production in microdissected tubules, a large number of hormones were screened to determine which may regulate cAMP production. Arginine vasopressin stimulated cyclic AMP production in both the initial and terminal IMCD. Isoproterenol and other beta-adrenergic agents increased cyclic AMP production only in the initial IMCD, which is the only part of the IMCD that possess intercalated cells. Epinephrine, working through alpha-2 adrenergic receptors, substantially inhibited vasopressin-stimulated cyclic AMP production. Epinephrine also inhibited urea permeability in isolated perfused IMCD segments. Maeda and Knepper have recently begun to measure intracellular calcium using FURA-2 in unperfused IMCD segments as a means of determining which hormones increase intracellular calcium through activation of the phosphoinositide signalling pathway. Arginine vasopressin was found to trigger a rise in intracellular calcium with a dose-response relationship parallelling vasopressin-mediated rises in cyclic AMP production. The rise in intracellular calcium occurs with the V-2 selective agonist dDAVP and is not blocked by V-1 antagonists, suggesting that occupation of V-2 receptors may be associated with both activation of the phosphoinostide pathway and adenylate cyclase. These studies will be extended to screen a large number of potential regulators of sodium chloride, urea, and water transport in the terminal IMCD.

Nielsen and Knepper are investigating mechanisms by which vasopressin increases urea and water permeability in the terminal IMCD of rats. Kinetic studies of urea and water permeability changes following addition and withdrawal of vasopressin reveal similar kinetics for the two permeabilities. Both display a two-phase increase with a rapid initial rise in

permeability followed by a slower secondary increase. With vasopressin withdrawal, both display a two-phase decrease with a rapid initial decrease followed by a slower secondary fall. Mathematical modelling of this process indicates that these kinetics can best be explained by the assumption that vasopressin has two effects: 1) to increase the kinetic rate constant for activation/exocytosis and 2) to decrease the kinetic rate constant for inactivation/endocytosis. When endocytosis was inhibited by reversal of the imposed osmotic gradient to a lumen>bath orientation, the decrease in water permeability following vasopressin withdrawal was entirely prevented. However, the same maneuver had no effect on urea permeability reversal following vasopressin withdrawal. This suggests that urea permeability reversal may not depend on endocytosis, although this conclusion will need verification by other methods. Electron microscopy using fluid phase markers (collaboration with J. Muller) has demonstrated that the withdrawal of vasopressin is associated with rapid endocytosis. In addition, there is an associated disappearance of coated pits in the IMCD apical membrane and a disappearance of intramembrane particles in the apical membrane (collaboration with J. Wade). Both of these structures are thought to contain water channels, based on studies presented by others. Preliminary results indicate that the incidence of intramembrane particle clusters remains high when vasopressin is removed in the presence of a lumen>bath gradient which correlates with the maintenance of a high water permeability. Current plans include development of reliable methods for measurement of endo- and exocytosis in perfused tubules and collaboration with Hediger (at Harvard) and Agre (at J. Hopkins) to clone cDNAs for and raise antibodies against the vasopressin-regulated urea carrier and the vasopressin-regulated water channel. These tools will be essential in the definition of the processes involved in transport regulation by vasopressin.

Chou and Knepper are continuing their studies of the permeability properties of the thin limb segments of the chinchilla loop of Henle. The descending limb of long-loops is highly permeable to water over the first 80% of its length. In contrast, the final 20% has a relatively low water permeability. To establish a correlation between these properties and the structures of the perfused segments, water permeability was measured in the perfused segments and then the tubules were fixed for transmission electron microscopy. Results thus far indicate that the upper and middle parts of the descending limb are lined by Kriz type II and type III thin limb epithelia, respectively. Although the tubules with type III architecture have slightly lower water permeabilities, in general all type II and III tubules had relatively high water permeabilities. The tubules from the distal 20% of the descending limb again were found to have low osmotic water permeabilities, and the epithelial architecture was consistent with a Kriz type IV epithelium, which is generally associated with the ascending thin limb. Thus, the low permeability segment appears to be pre-bend ascending limb, although we do not yet have enough data to tell whether the structure of the low water permeability descending limbs differs from that of the ascending limb.

Nielsen and Knepper (in collaboration with Agre) have carried out immunocyto-chemical studies to localize the CHIP28 water channel protein along the rat nephron. CHIP28 was found to be exclusively localized to the proximal tubule and thin descending limbs and was

present in both apical and basolateral membranes. It is hypothesized that the presence of CHIP28 is responsible for the high water permeability of the type II and III descending limb segments. Chou and Knepper have also measured urea and NaCl permeabilities of the long-loop segments. Both permeabilities increase progressively along the length of the descending limb toward the papillary tip. The high permeabilities in the outer portion of the inner medullary descending limb confirm findings of Imai that appear to rule out a role for the passive model of Kokko and Rector. Recent modelling studies in collaboration with Layton (at Duke) demonstrate anew that the high descending limb permeabilities make the passive model infeasible. The high urea permeability is not phloretin-sensitive and the permeabilities of the epithelia to other solutes increase along the descending limb in parallel with urea and NaCl. Based on these results we speculate that permeation through the tight junctions may be responsible for the high permeabilities. We plan studies to determine whether factors known to modulate junctional permeabilities may reduce solute permeabilities into a range that is compatible with the passive model. If these experiments fail to demonstrate low solute permeabilities in the descending limb, then further studies will be targeted toward other concentrating hypotheses including the idea that the energy for concentration may derive from the mechanical force of the renal pelvic contractions.

Flessner, Mejia, and Knepper have recently completed a series of studies aimed at determining the permeability of loop of Henle segments to bicarbonate, NH^{4+}, and NH_3, species important in the acid-base excretory role of the renal medulla. The permeability of the outer medullary part of the long-loop descending limb to both NH^{4+} and NH_3 were relatively high, indicating that ammonium that is actively transported out of the thick ascending limb can enter the neighboring descending limb resulting in countercurrent multiplication of ammonium in the renal medulla. Associated mathematical modelling studies by Mejia, Flessner, and Knepper indicate that the well-known phenomenon of alkalinization of descending limb fluid in the rat kidney can be accounted for largely by the predicted entry of NH_3 in the outer medullary descending limb. Flessner and Knepper demonstrated that the ascending thin limb is highly permeability to NH^{4+}, thus identifying a putative route of ammonium egress from the loop of Henle in the inner medulla and a source of ammonium for secretion into the neighboring inner medullary collecting ducts. Flessner and Knepper also demonstrated that the rat medullary thick ascending limb has an extremely low permeability to NH_3 (as shown previously by Garvin et al. in the rabbit). This low permeability is necessary to the overall mechanism of ammonium absorption from the thick ascending limb since it prevents NH_3 backflux which would otherwise subvert the net active absorption of ammonium. Mejia and Knepper are currently developing a large scale mathematical model of the acid-base transport function of the kidney. This model will be used to test hypotheses formulated over the last few years pertaining to the countercurrent multiplication of ammonium in the medulla and the disposal of bicarbonate in the renal medulla.

An important factor in the regulation of ammonium excretion and thus the regulation of systemic acid-base balance is the regulation of ammonium production in the proximal tubule. Studies by Wright, Packer, Garcia-Perez, and Knepper have demonstrated that the

activity of the ammoniagenic enzyme glutamic dehydrogenase (GDH) in the rat renal cortex increases gradually over at least 7 days after initiation of systemic acid loading. This increase in activity lags the measured increase in ammonium excretion which occurs largely within the first 24 hours. The rise in GDH activity was associated with a 3-fold rise in GDH mRNA as judged from Northern blots. DiGiovanni, Madsen, and Knepper have utilized the same animal model to study changes in glutaminase and GDH activities in microdissected rat S-1 segments (which have been shown to be associated with the greatest rise in ammonium secretion in response to acid-loading of rats in vivo, relative to other micropuncturable segments). The S-1 segment exhibited a sharp increase in glutaminase activity after day one of the treatment, corresponding to the rapid increase in ammonium excretion seen in the same animals. The glutaminase activity continued to increase through day four. In contrast, GDH activity did not increase on day one and in fact even after day four there was little or no increase in activity. This result confirms the observation of Wright et al. that GDH activity tends to increase relatively slowly and modestly in response to acid loading. Currently, the same investigators are studying relative GDH mRNA levels in the S-1 segments using reverse transcription and polymerase chain reaction in single tubules to amplify the specific glutaminase mRNA signal. These experiments will address whether glutaminase mRNA rises in this segment prior to the rise in enzyme activity. Similar techniques are proposed to measure glutaminase mRNA stability in order to test the hypothesis of Curthoys that the rise in glutaminase mRNA in response to acidosis could be due to increased glutaminase mRNA stability.

Regulation of water permeability

Flamion and Spring continued their work with a computer controlled, video, light microscope technique to measure the size and shape of the cells in isolated perfused rat medullary collecting ducts. By following the rate of change of cell volume in the first seconds after a step change in the concentration of the bathing solutions, they measured the osmotic water permeability of the cell membranes. The effects of antidiuretic hormone (ADH) and water deprivation on water permeability were also determined. These are the first direct measurements of this important parameter in medullary collecting ducts.

Transport in model epithelia

The regulation of ionic pathways by a variety of arachidonic acid metabolites was studied by Kersting and Spring. They characterized the changes in ion transport caused by inhibition of the production of arachidonic acid metabolites in cultured human pancreatic cells with and without the cystic fibrosis genetic defect. They were able to rectify the defect by treatment of the cells with inhibitors of the production of epoxygenase metabolites of arachidonic acid. These studies indicate an important role for arachidonic metabolites in the control of ion transport involved in cystic fibrosis.

Harris, Chatton, and Spring have developed the instrumentation and methods to study the composition of the fluid filling the spaces between epithelial cells. They utilize cultured

renal cells (MDCK as well as LLC-PK_1), grown on glass coverslips to measure the pH of the spaces between the cells and to determine the diffusion coefficient of fluorescent dyes within these spaces.

Regulation of fluid flow in the eye

Zadunaisky and Spring have been studying human ocular trabecular meshwork cells in tissue culture. Two cell lines are under study--one from a normal eye and one from a patient with glaucoma. The relationship between the extent of contraction of the cells and the dose of a variety of drugs used in the treatment of glaucoma has been investigated.

Organic osmolytes

Bacterial, plant, and invertebrate animal cells are known to accumulate compatible, osmotically active, organic intracellular solutes when their environment becomes hyperosmotic. These organic "osmolytes" help maintain the intracellular milieu because they do not perturb vital intracellular macromolecules, in contrast to sodium and potassium salts which in abnormally high concentrations do perturb macromolecules. Most mammalian body fluids are not normally hyperosmotic and the cells exposed to them do not normally express organic osmolytes. The exception is the renal inner medulla in which the interstitial fluid is hyperosmotic to a variable extent because of the renal concentrating mechanism. We identified large and variable amounts of organic osmolytes in rat and rabbit inner medullary cells, namely sorbitol, inositol, glycerophosphorylcholine (GPC), taurine, and betaine.

Control of the cellular accumulation of these osmolytes is most readily studied in tissue culture. We screened several renal cell lines in hyperosmotic media and found that cells that survived accumulated the same organic osmolytes previously found in intact renal medullas. These cell lines are now being studied in detail. The findings with regard to osmotic regulation of the individual organic osmolytes are, as follows:

A. Sorbitol accumulates in GRB-PAP1 cells by synthesis from glucose, catalyzed by aldose reductase. Hypertonicity increases aldose reductase gene transcription, mRNA abundance, and protein abundance. We have cloned the rabbit aldose reductase gene and are testing the 5' flanking region in a transient expression system to determine the molecular mechanism by which hypertonicity stimulates transcription of this gene.

B. Inositol accumulates in renal medullary cells when medium NaCl is increased. The mechanism is increased transport of inositol into the cells from the medium. We have cloned the cDNA for the inositol transporter by expression in toad oocytes. Using the cDNA as a probe, we find that high NaCl increases the amount of inositol transporter mRNA. Future studies will investigate the molecular mechanism by which high NaCl increases inositol transporter gene expression.

C. Betaine, like inositol, is accumulated by MDCK cells in high NaCl medium by increased transport into the cells. We have cloned the cDNA for the betaine transporter by expression in toad oocytes. Using the cDNA as a probe, we find that high NaCl increases the amount of betaine transporter mRNA. The betaine transporter has high sequence similarity to several transporters from brain but not to other transporters. Thus, a new transporter gene family has been discovered. Future studies will investigate the molecular mechanism by which high NaCl increases betaine transporter gene expression.

D. GPC. In contrast to the other osmolytes, GPC accumulation is triggered by high urea, as well as by high NaCl. Accumulation of GPC by MDCK cells in hyperosmotic medium is due to increased net synthesis. Choline is an essential precursor. There is a small decrease in the rate of GPC synthesis in hyperosmotic medium, and a larger fall in GPC degradation. Measurements of the enzymes involved in synthesis (phospholipase A_2) and degradation (GPC: choline diesterase) are in agreement. Thus, the accumulation of GPC is due to a predominant decrease in the rate of degradation. In order to investigate the mechanism by which urea or salt inhibits GPC:choline diesterase, we have purified the enzyme and will measure the amino acid sequence, prepare antibodies, and clone its cDNA.

In addition, we now find that caffeine increases GPC both at normal and elevated osmolality. There is a large increase in phospholipase A_2 activity that is prevented by ryanodine. Since the ryanodine receptor of muscle and brain was not found to be expressed in kidney, a novel system may be involved.

E. Interactions between osmolytes. In order to test whether accumulation of one osmolyte in hypertonic medium might affect the accumulation of the others, we have systematically varied the concentrations of their metabolic and transport substrates. Generally, when accumulation of one is altered experimentally, one or more of the others changes reciprocally. In order to investigate the mechanisms involved, we are measuring mRNAs for the various effector molecules under these conditions.

Accumulation of organic osmolytes in response to osmotic shock is a basic biological phenomenon previously identified in virtually all cells from bacteria to those in lower vertebrates. The present recognition of its vital role in renal medulla is the first indication that the accumulation is more than a curiosity in mammalian cells. Possible disorders of this system have not yet been systematically investigated, but there are a number of poorly understood diseases of the renal medulla that should be considered. Along this line, we have preliminary evidence that renal organic osmolytes are affected by the same non-steroidal analgesics that cause renal medullary necrosis. Further, the aldose reductase system, whose function is being elucidated in the renal medulla, is implicated in complications of diabetes in eyes, nerves, and kidneys.

Annual Report
Laboratory of Molecular Cardiology
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The Laboratory of Molecular Cardiology investigates the regulation, expression and function of contractile proteins in muscle and nonmuscle cells. We are particularly interested in the mechanisms responsible for regulating the contractile activity of smooth muscle and nonmuscle cells as well as the factors that govern the expression of the genes encoding the contractile proteins. In addition, we have initiated a program to study a particular set of homeobox and pou genes that play a role in the early development of Drosophila and mammalian embryos. These studies are directed towards understanding the mechanism of action and regulation of the genes involved in neuromuscular and cardiac development. By studying the genes, mRNA and proteins involved in these various processes, we hope to understand the mechanisms by which cells differentiate, alter their phenotype, migrate, change their shape, move their membrane receptors, secrete cellular products and proliferate. We plan to use this information to understand both normal and disease processes.

Below is a summary of the various projects under study during the past year. Of particular note during the past year was: 1) the detection of an altered isoform of slow skeletal muscle myosin in the muscle of patients suffering from Hypertrophic Cardiomyopathy; 2) the discovery of inserts of cassettes of amino acids in the head region of the nonmuscle and smooth muscle myosin heavy chains, near to the ATP and actin binding sites, and 3) evidence that an insertion of 7 amino acids in the head region of both the 204 kD and 200 kD MHC of gizzard smooth muscle myosin may alter its biological properties compared to smooth muscle isolated from the aorta; 4) identification of the transcriptional start site for the gene encoding nonmuscle myosin heavy chain-A and characterization of the surrounding regulatory sequences; 5) the use of a combination of experimental and modeling data to gain further insight into the mechanism underlying the efficiency of smooth muscle contraction compared to skeletal muscle; 6) initiation of a new program to study the function and regulation of three homeobox genes known to be involved in Drosophila embryogenesis, which appear to play a role in muscle cell differentiation and neuromuscular synaptogenesis.

Growth and Differentiation of Smooth Muscle and Nonmuscle Cells (S. Kawamoto, Z01 HL 01665-17 MC). At least two genes located on two different chromosomes are known to encode nonmuscle myosin heavy chain II in humans. The purpose of this study is to understand the mechanisms responsible for regulating the expression of these two genes. To accomplish this, the 5' portion of the human nonmuscle myosin heavy chain-A gene, including the promoter region, was cloned and studies to characterize this portion of the gene were initiated. The results show that a major and a minor transcriptional start site could be identified by both primer extension and RNase protection analysis using mRNA from human leukemia (Jurkat) and epidermoid carcinoma (A431) cells. In keeping with the findings from other housekeeping genes, the regions both upstream and downstream from the transcriptional start site were found to be rich in GC, but were also found to lack an apparent TATA box. Using the luciferase construct as a reporter, a fragment of approximately 175 bp (115 bp upstream and 60 bp downstream from the major transcriptional start site) was found to possess core promoter activity. Including a fragment of DNA approximately 150 bp further downstream results in an approximately 10-fold increase in transcriptional activity in fibroblasts. In contrast, this downstream sequence does not activate transcription in differentiated skeletal muscle cells, in which endogenous mRNA for this gene is also down-regulated. A gel shift assay using nuclear extracts from NIH 3T3 cells and C2C12 myotubes demonstrated that the downstream 150 bp DNA fragment forms a different type of complex with NIH 3T3 nuclear protein compared to nuclear protein isolated from C2C12 myotubes. Present studies are directed to identification of the DNA binding site of the NIH 3T3 nuclear protein in the downstream DNA fragment.

Myosin and Caldesmon Phosphorylation in Nonmuscle Cells (G. Bazile, J.R. Sellers, E.V. Harvey, Z01 HL 01785-13 MC). Caldesmon is a calcium-calmodulin binding protein that appears to play a role in the regulation

a variety of cells under normal and pathological conditions, we hope to gain insight on the function of these two molecules.

Cloning of the cDNAs Encoding Neuronal Myosin Heavy Chains (M. Takahashi, S. Kawamoto, R.S. Adelstein, Z01 HL 04208-06 MC). During the cloning of the cDNA encoding chicken brain nonmuscle myosin heavy chain-B, we found a 63 nucleotide insertion encoding 21 amino acids in the head region of the myosin heavy chain near to the actin binding site and a 30 nucleotide insertion encoding 10 amino acids near to the ATP binding site. Analysis using S1 nuclease showed that both inserts were expressed in a tissue-dependent manner. That is, mRNA containing the inserts is present in tissues of the nervous system, but is absent from other nonmuscle cells, all of which contain the non-inserted isoform of myosin heavy chain-B. Similar inserts were also found in corresponding positions in human cerebellar mRNA. Of note is the presence of a cdc2 kinase site in the sequence located near to the ATP binding site. A peptide constructed based upon this 10 amino acid sequence was able to serve as a substrate for cdc2 kinase. Antibodies raised against a peptide synthesized based upon the 21 amino acid insert found near to the actin binding site recognized a MHC isoform in the same tissues that are enriched for the mRNA. These insertions appear to be a mechanism for generating additional myosin heavy chain isoforms specific to the nervous system. Of particular interest is the recent identification by Naina Bhatia-Dey of a similar insert near to the ATP binding site in Xenopus oocytes (see below, Z01 HL 04222-01). Since these inserted sequences are located near to the ATP and actin binding sites, they may play an important role in regulating the actin-activated MgATPase activity of myosin.

Myosin Phosphorylation and the Regulation of Contractile Activity (C. Kelley, R.S. Adelstein, W. Anderson, Z01 HL 04210-05 MC). The identification of inserted segments of amino acids near to the ATP binding site in the chicken brain nonmuscle myosin heavy chain isoform suggested that these sequences might exist in other myosins, too. Using mRNA prepared from both chicken gizzard smooth muscle as well as chicken aorta smooth muscle and the reverse transcriptase-polymerase chain reaction, an insert of 21 nucleotides encoding 7 amino acids was detected in the gizzard smooth muscle myosin heavy chain, but not in the aortic smooth muscle myosin heavy chain (M. Takahashi and J. Yu, this lab). Moreover, this insert was found to be present both in the 204 kD myosin heavy chain as well as the 200 kD myosin heavy chain of chicken gizzard smooth muscle. cDNA sequencing by a number of other laboratories as well as RT-PCR scanning of the myosin heavy chain by our laboratory failed to detect any other differences in the myosin heavy chain between gizzard and aorta. Differences in biological activity between the gizzard and aorta myosin were measured by the actin-activated MgATPase activity as well as by an in vitro motility assay. We found a 2-fold increase in both the V_{max} of the actin-activated MgATPase activity as well as in the velocity of movement of the actin filaments in the in vitro motility assay of gizzard myosin compared to aorta. SDS-polyacrylamide gel electrophoresis and polyacrylamide gel electrophoresis in the presence of urea as well as isoelectric focusing revealed that there were two isoforms of the 17 kD light chain in aorta, only of one of which was found in the gizzard. Exchange of the gizzard 17 kD light chain onto the aorta myosin heavy chain resulted in no change in the in vitro motility assay suggesting that the isoforms of the 17 kD light chain were not responsible for the increase in the actin-activated MgATPase activity nor in the differences in the in vitro motility assay. Our results suggest that the enzymatic activity of myosin from a phasic intestinal muscle is greater than that of myosin from a tonic vascular-type muscle and that this difference may be due to the insertion of the 7 amino acids near to the ATP binding site in the intestinal myosin heavy chain.

Characterization of Myosin I (J.R. Sellers, E.V. Harvey, Z01 HL 04212-04 MC). Two different myosin I molecules are under study. One of these myosin I molecules has been isolated from chicken intestinal epithelial brush border and contains a heavy chain of 110 kD with three bound calmodulin molecules. This myosin does not form filaments, but does have an actin-activated MgATPase activity and translocates actin filaments in an in vitro motility assay. Motility and actin activation of the MgATPase activity are inhibited at high calcium concentrations due to a dissociation of a fraction of the calmodulin molecules. Re-addition of calmodulin restores the motility and the actin-activated MgATPase activity. Interestingly, tropomyosin bound to the actin filaments also inhibits motility and the actin-activated MgATPase activity and decreases the affinity of actin for myosin. In order to study the motor responsible for cytoplasmic streaming in Nitella, recent efforts have been directed towards isolating a putative

myosin I from this freshwater green alga. Preliminary characterization of the movement of actin filaments by a crude extract revealed that the velocity varies from 5-60 um/sec which makes this the fastest motor myosin yet studied. The extract from Nitella supports movement of actin filaments even in the presence of calcium ions unlike purified brush border I myosin from chicken intestinal cells. These studies are being carried out in a collaboration with Dr. Paul Matsudaira and Kathy Collins (Whitehead Institute, MIT) and Bechara Kachar (NIDCD).

Interaction of Invertebrate Myosin With Actin (F. Wang, J.R. Sellers, E.V. Harvey, W. Anderson, Z01 HL 04213-03 MC). Two separate studies concerning the mechanism underlying the regulation of invertebrate myosins are being carried out. The first involves the regulation of the horseshoe crab, Limulus, which is the first striated muscle described to have both a myosin-based and an actin-based regulatory system. In vitro experiments show that Limulus myosin needs to be phosphorylated in order to propel actin filaments in a motility assay. When both troponin and tropomyosin are bound to the actin filaments, calcium ions are required for the movement of the actin filaments over phosphorylated myosin. However, actin cannot be propelled even in the presence of troponin, tropomyosin and calcium, if the Limulus myosin is dephosphorylated. These results demonstrate that, in Limulus, the "off" state of either the thin filament or the thick filament regulatory system is dominant. Partial sequence of the tryptic phosphopeptides of the Limulus myosin light chains generated following phosphorylation by gizzard myosin light chain kinase shows that the sequence around the phosphorylated site in both the 21 kD and 31 kD myosin light chain is more similar to that of the vertebrate smooth muscle myosin light chain than it is to the vertebrate striated muscle myosin light chain. This observation is keeping with the important regulatory role that phosphorylation appears to play. Despite a wealth of information about Lethocerus flight muscle mechanical properties and structure, very little is known about the regulation of this insect muscle. Therefore, myosin was isolated from Lethocerus flight muscle and an in vitro motility assay used to examine the regulatory properties of the sliding velocity of actin filaments over the myosin. The results show that the myosin-linked regulatory system may play an important role in Lethocerus flight muscle. The light chains of myosin made from this muscle can be phosphorylated by gizzard myosin light chain kinase or by an endogenous kinase present in this muscle. Both phosphorylations stimulate movement of actin filaments over myosin.

Myosin Phosphorylation in Human T-lymphocytes (M. Moussavi, R.S. Adelstein, W. Anderson, Z01 HL 04216-02 MC). Phosphorylation of the myosin heavy chain as well as the myosin light chain is being studied in myosin from a number of different cells and sources, including human T-lymphocytes grown in culture, lymphocytes isolated from healthy donors, as well as myosin purified from bovine brains. Previous work has shown that the myosin heavy chain in human lymphocytes is a mixture of both myosin heavy chain-A and B isoforms with the A form predominating, whereas the myosin isoform isolated from brain is predominantly the B isoform. Treatment of ^{32}P-labeled T-lymphocytes (Jurkat cells) with phorbol ester resulted in incorporation of phosphate into both the myosin heavy chain and the 20 kD myosin light chain. Isoelectric focusing of the tryptic myosin heavy chain phosphopeptides showed the presence of a new phosphopeptide in PMA treated cells that migrated with a peptide known to be produced by protein kinase C phosphorylation. On the other hand, short-term treatment of normal human lymphocytes with IL-2 resulted in phosphorylation of the myosin heavy chain at a different site, best correlated with a peptide known to be phosphorylated by casein kinase II. When these cells are treated with phorbol ester, isoelectric focusing of the tryptic phosphopeptides resulted in a pattern that was identical to that generated from Jurkat cells. These results suggest that short-term stimulation of normal lymphocytes with IL-2 was not associated with phosphorylation of myosin by protein kinase C. When the myosin heavy chain phosphopeptides produced following in vitro phosphorylation of bovine brain myosin (myosin heavy chain-B isoform) by protein kinase C are compared with those produced from human platelet myosin (myosin heavy chain-A isoform), there are significant differences. Preliminary results suggest that the brain myosin heavy chain-B isoform contains protein kinase C phosphorylatable sites in the head portion of the molecule which are not present in the myosin heavy chain-A isoform. This work was carried out with the collaboration of J. Siegel (FDA) and P. McPhie (NIDDK).

Structure and Function of Cardiac Myosin (G. Cuda, J.R. Sellers, Z01 HL 04217-02 MC). The purpose of this study is to define the enzymatic properties of a myosin purified from the slow skeletal muscle of patients with Hypertrophic Cardiomyopathy (HCM). Recently, several missense mutations have been identified in the gene

encoding the beta myosin heavy chain isoform, which is expressed both in cardiac as well as skeletal muscle. Soleus muscle biopsies were obtained from both normal controls and HCM patients carrying two distinct beta myosin heavy chain missense mutations. One was an $Arg_{403}Gln$ mutation, and the second was a $Leu_{908}Val$ mutation. Myosin purified from these samples was probed using the sliding filament actin motility assay, where the rate of translocation of actin filaments moving over myosin monomers bound to a nitrocellulose-coated surface is measured. In myosin prepared from both mutations, the speed of the actin filament sliding was markedly decreased with respect to normal controls. To identify the presence of the mutated beta myosin heavy chain isoform, myosin purified from soleus muscle biopsies in the patients carrying the $Arg_{403}Gln$ mutation was digested with the arginine-specific endoprotease Arg-C. An antibody previously raised against a synthetic peptide flanking the mutated residue was able to recognize an aberrant 6.5 kD peptide in Western blots which resulted from the loss of Arg residue 403. In addition, the expected 3.5 kD peptide was seen since the patient is heterozygous for the abnormal gene. As expected, only the 3.5 kD peptide was found in the myosin from a normal control. These studies were carried out with the cooperation of Neal Epstein and Lameh Fananapazir (NHLBI).

Expression of Nonmuscle Myosin Heavy Chain cDNA in Vertebrate Cells (H.F. Elson, R.S. Adelstein, Z01 HL 04218-02 MC). A cDNA fragment 2.8 kb in size encoding 93 kD of the myosin rod polypeptide and 0.3 kb of the 3' untranslated region was transfected into C2C12 cells following its insertion in both the sense and antisense direction in the pMAMneo vector. This expression vector contains an inducible promoter and the selectable neomycin gene conferring G418 resistance. C2C12 cells can undergo differentiation into fused myotubes following their transfer from 20% fetal bovine serum to 2% horse serum. Studies on mass transfected cultures with the sense construct showed no changes in the morphology of the cultured cells. In contrast, mass cultures transfected with the antisense construct and grown in 20% fetal bovine serum show that a small proportion of the cells assumed a rounded, flattened discoid morphology, with many micronuclei or coalesced nuclei. We speculate that a deficiency in the nonmuscle myosin heavy chain may result in the cell flattening and multinuclearity seen in these cells, but, as of yet, have been unable to demonstrate a decrease in the nonmuscle polypeptide in these cells. (The relatively small number of abnormal cells makes this quantitation difficult.) When cells transfected with the antisense construct are changed to differentiation conditions, myoblast fusion appears to be inhibited and SDS gels revealed an altered polypeptide composition, but no clear change in the myosin isoforms.

Expression and Site-directed Mutagenesis of Nonmuscle Myosin Heavy Chains (R.S. Adelstein, Y.A. Preston, Z01 HL 04219-02 MC). We have succeeded in expressing the head fragment of the chicken brain nonmuscle myosin heavy chain using the baculovirus expression system. We cloned cDNA encoding the first 1200 amino acids, starting at the amino-terminus of the myosin heavy chain, into a vector that is capable of undergoing homologous recombination with the wild-type baculovirus when transfected into intestinal cells of the army fall worm. Expression of the purified recombinant virus in these cells results in the production of mg quantities of a polypeptide, not seen in control cells, which encodes the amino-terminal end of the nonmuscle myosin heavy chain-B molecule. The identity of this polypeptide was confirmed by Western blotting using a monoclonal antibody recognizing the head region of the myosin molecule. When the expressed form of the myosin heavy chain was partially purified on molecular sieve chromatography, it only demonstrated 10% of the expected ATPase activity. This was attributed to the misfolding of the nascent myosin heavy chain and experiments are presently underway to express the 17 kD myosin light chain using the same baculovirus expression system. The two recombinant viruses, one expressing the myosin heavy chain and one expressing the myosin light chain will then be used to coinfect insect cells in an effort to produce a fully active myosin molecule. This construct can then be manipulated using the polymerase chain reaction to introduce both single amino acid mutations as well as to insert cassettes of amino acids into the head region of the myosin molecule (see Z01 HL 04208-06). These mutated forms of the myosin heavy chain will be used to identify those amino acid residues that contribute to myosin's biological activity.

Identification of Chicken Pou Genes (M.B. Goens, Y.S. Kim, R.S. Adelstein, Z01 Hl 04220-01 MC). We have initiated studies to clone cDNA encoding the Pou domain protein which may be involved in chicken heart development. Most Pou domain genes identified to date are expressed in the nervous system. The mesoderm of the great vessels and the nerves innervating the heart are derived from the neural crest. Therefore, Pou genes could

be involved in cardiac development. In an effort to clone cDNA encoding a Pou gene that plays a role in cardiac development, chicken heart and embryonic cDNA and chicken genomic libraries were screened, using degenerate PCR primers derived from the most conserved area of the Pou domain amino acid sequence. This sequence is conserved across species and class of the Pou proteins. We have also used reverse transcriptase and the same PCR primers to obtain a chicken homolog of the Pou gene using chicken brain mRNA as template. The various PCR reactions yielded 350 bp fragments as predicted by the Drosophila Pou sequence. These fragments were subcloned into BluescriptSK+ and are now being sequenced. If any chicken Pou genes are identified, we will explore the timing and distribution of their expression in chick embryos by in situ hybridization. We will also use these clones to rescreen stage-specific chicken embryonic cDNA libraries.

Function of Drosophila NK-homeobox Genes in Mesodermal Cell Differentiation (Y.S. Kim, S.J. Kim, Y.M. Lee, Z01 HL 04221-01 MC). Recently, a novel Drosophila homeobox gene cluster located on the right arm of the 3rd chromosome was discovered (Kim and Nirenberg, PNAS 86, 7716, 1989). The function of these NK-homeobox genes are presently under study. Characterization of gene structure and in situ hybridization analysis suggests that these homeobox genes may be involved in mesodermal cell differentiation and muscle segment formation in the Drosophila. Particularly, it was shown that these genes are expressed in embryonic mesodermal cells and neurons and specific muscle fragments. The function and regulation of these genes (i.e., NK-1, NK-3 and NK-4) is being studied during Drosophila embryogenesis with a special emphasis on neuromuscular synaptogenesis and muscle cell differentiation. These studies include use of target gene cloning, mutant analysis generated by P element-mediated mutagenesis and the use of transgenic flies. Learning how the NK-homeobox genes function in early embryogenesis should increase our understanding of the fundamental processes of cellular differentiation.

cDNA Cloning and Characterization of Nonmuscle Myosin From Xenopus Laevis (N. Bhatia-Dey, R.S. Adelstein, Z01 HL 04222-01 MC). cDNA encoding the entire nonmuscle myosin heavy chain from Xenopus Laevis were cloned by N. Bhatia-Dey in collaboration with I. Dawid (LMG, NICHD). The sequence showed remarkable identity to the nonmuscle myosin heavy chain-B isoform previously cloned from chicken and human cDNA libraries in this laboratory. Messenger RNA from the Xenopus gene was found to be expressed throughout development starting from unfertilized eggs to the swimming tadpole stage. In adult tissues the mRNA is relatively abundant in lung, heart and brain. Using a peptide antibody raised against the myosin heavy chain-B isoform, based on the chicken sequence, immunochemical localization was carried out and showed that the nonmuscle myosin heavy chain is expressed throughout the entire embryo during embryogenesis. Of particular interest was the presence of a 16 amino acid insertion in the area of the myosin heavy chain ATP binding site, which shares a number of amino acid identities with a similar insertion in the head portion of the chicken nonmuscle myosin heavy chain-B isoform (Takahashi et al., J. Biol. Chem., in press 1992). Studies are underway to determine whether, similar to the chicken, there are two isoforms present in the Xenopus, an inserted and a non-inserted one, or whether only the inserted isoform is present.